A STUDY OF THE EFFECT OF CLEISTANTHIN C ON BLOOD PRESSURE OF RATS AND THE EFFECTIVENESS OF VASOPRESSORS TO CONTROL HYPOTENSION IF IT OCCURS.

A Dissertation submitted in partial fulfillment of the requirement

for the Degree of Doctor of Medicine in Physiology (Branch – V)

Of The Tamilnadu Dr. M.G.R Medical University,

Chennai -600 032

Department of Physiology

Christian Medical College, Vellore

Tamilnadu

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This is to certify that the thesis entitled **"A study of the effect of Cleistanthin C on blood pressure of rats and the effectiveness of vasopressors to control hypotension if it occurs."** is a bonafide, original work carried out by Dr. Sajal Clarence Singh, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamilnadu Dr. M.G.R Medical University, Chennai to be held in April- 2017.

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Principal,

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**A study of the effect of Cleistanthin C on blood pressure of rats and the effectiveness of vasopressors to control hypotension if it occurs.**" were carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

Dr. Sajal Clarence Singh,

Department of Physiology,

Christian Medical College,

Vellore – 632 002

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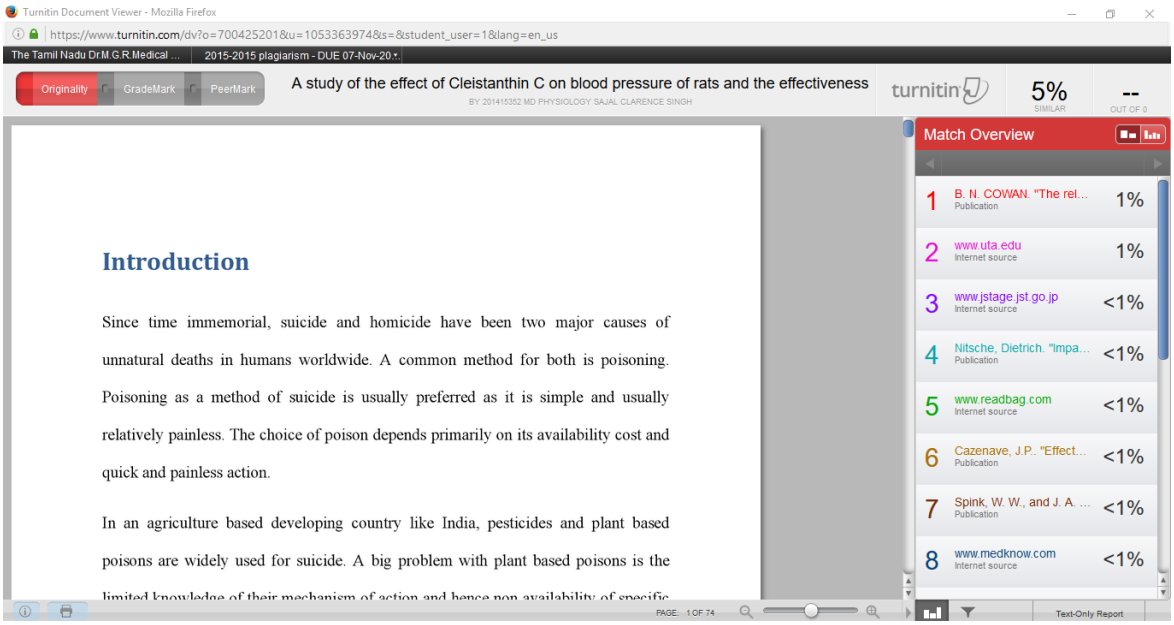
Introduction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Introduction
Since time immemorial, suicide and homicide have been two major causes of unnatural deaths in humans worldwide. A common method for both is poisoning. Poisoning as a method of suicide is usually preferred as it is simple and usually relatively painless. The choice of poison depends primarily on its availability cost and quick and painless action.
In an agriculture based developing country like India, pesticides and plant based poisons are widely used for suicide. A big problem with plant based poisons is the
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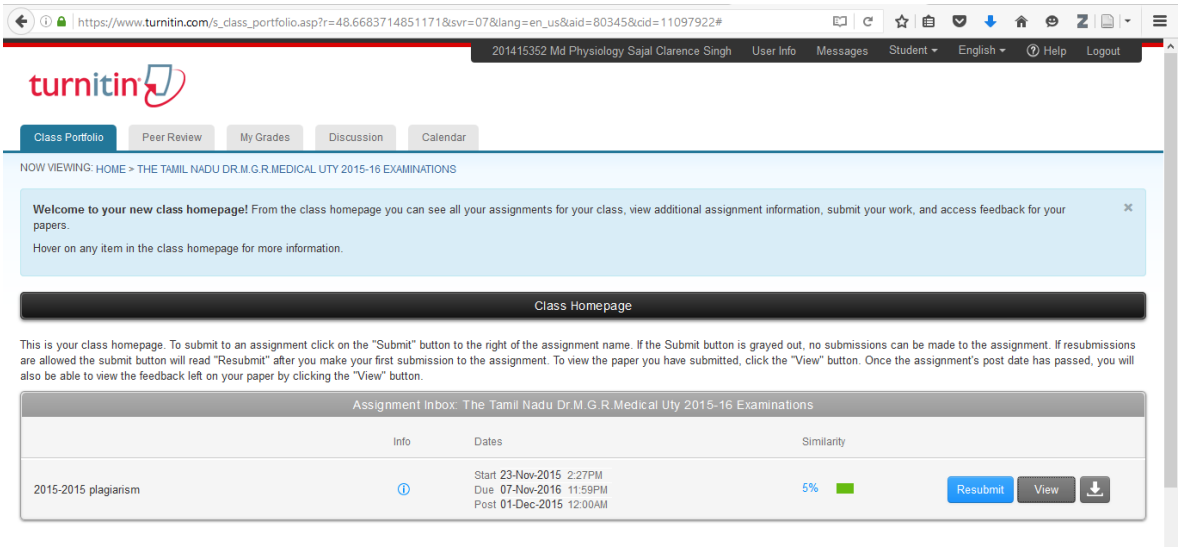
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Abstract

A common mode of suicide in rural south Indian states of Tamil Nadu and Puducherry is consumption of leaves of a poisonous shrub *Cleistanthus collinus*. Intractable hypotension unresponsive to vasopressors is stated as one of the major cause for death in *C.collinus* poisoning. In addition to adrenaline and nor adrenaline, phenylephrine is also used in the treatment regimen of shock. In our lab, we have identified a new signaling pathway, where phenylephrine a pure alpha agonist produced vasodilatation in the presence of high levels of nitric oxide . Here, we sought to investigate the use of vasopressor like phenylephrine improves or worsens *C.collinus* induced hypotension in an animal model. Since, Cleistanthin C was found to be the major toxin in the fresh leaf boiled extract of *C.collinus*, experiments were performed with cleistanthin C.

Aim: To study the effect of Cleistanthin C on blood pressure of rats and the effectiveness of vasopressors to control hypotension if it occurs.

Objectives:

1. To study the effect of intraperitoneal administration of Cleistanthin C on blood pressure of rats.
2. To study the effect of intravenous administration of Cleistanthin C on blood pressure of rats.
3. If hypotension occurs, to study the effect of administration of phenylephrine on the blood pressure of rats.

Methodology: Wistar rats were anaesthetized and administered the lethal dose of Cleistanthin C in 30% ethanol intraperitoneally in one group and intravenously in another group. Control group of each intervention group received 30% ethanol by the respective route of administration. Blood pressure was continuously recorded and data was acquired with CMC DAQ. If hypotension was seen, phenylephrine was administered by the same route of administration as the toxin. Animals were observed for 8 hours or till death whichever was earlier.

Result: In the intraperitoneal intervention group, even with 5 times the lethal dose of Cleistanthin C, no significant fall in blood pressure was observed. The control animals also did not show any fall in blood pressure.

In the intravenous intervention group, moderate drop in mean arterial pressure and a significant drop in diastolic blood pressure were observed. Addition of Phenylephrine led to significant drop in mean arterial pressure, systolic blood pressure, diastolic blood pressure and pulse pressure.

This indicates that Cleistanthin C alone very likely acts primarily on the resistance vessels. Administration of Phenylephrine to these rats, leads to vasodilatation of the larger arteries probably through a new signaling pathway.

Key words: Cleistanthus collinus, poisoning, cleistanthin C, refractory hypotension, Phenylephrine.

Introduction

Since time immemorial, suicide and homicide have been two major causes of unnatural deaths in humans worldwide. A common method for both is poisoning. Poisoning as a method of suicide is usually preferred as it is simple and usually relatively painless. The choice of poison depends primarily on its availability cost and quick and painless action.

In an agriculture based developing country like India, pesticides and plant based poisons are widely used for suicide. A big problem with plant based poisons is the limited knowledge of their mechanism of action and hence non availability of specific antidote. Moreover, the lack of knowledge of the mechanism of action also limits the use of diagnostic and monitoring modalities.

This study is aimed at studying the mechanism of action of poisoning with *Cleistanthus collinus* which is a poisonous plant used for homicidal and suicidal purpose in many parts of India especially southern states of puducherry and Tamil Nadu. The local name for this plant is Oduvanthazhai in Tamil. The victims are usually from the rural areas and have easy access to the plant. The poisoning is more common in women.

Cleistanthus collinus is a shrub from the Euphorbiaceae family of the plant kingdom. Leaves are commonly consumed for suicide although almost all parts of the plant are poisonous. The leaves of the plant maybe ground into a paste and consumed directly or mixed with other food items to make it more palatable. Another method of consumption is boiling the leaves in water and consuming the resulting decoction.

Onset of symptoms is slow and patients may be brought to the hospital as late as 96 hours post consumption of the poison. The symptom may include a combination of cardio respiratory, renal and neurological symptoms with gross metabolic derangements.

Clinical data as well as in *vivo* studies have shown evidence of type I distal renal tubular acidosis. This being more common in victims consuming boiled decoction. However this is not believed to be the cause of death in the victims as the victims who recover very often do still have alkaline urine at the time of discharge from the hospital.

Some studies have shown sudden respiratory arrest as the cause of death. Others have shown cardiac arrhythmias as the major cause of death. The most recent studies suggest that the death is due to refractory hypotension which does not respond to the usual vasopressors. Typically, death occurs between 3^{rd} and 7^{th} day. The death is sudden in a victim who was thought to be in recovery from the initial symptoms. In the absence of a specific antidote, the treatment is mainly symptomatic which include fluid replacement, electrolyte correction, cardiac pacing, oxygen and ionotropes.

In vivo animal studies also shown presence of type I renal tubular acidosis along with respiratory failure which ultimately leads to respiratory arrest and death.

The leaves of *Cleistanthus collinus* , which are the most common part of the plant implicated in poisoning, contains a cocktail of compounds each having properties of their own. Only few of these compounds are toxic while the others are non toxic. In

previous works on these leaves, 2 toxic compounds have been identified namely Cleistanthin A and Cleistanthin C. This study deals with the effect of Cleistanthin C on blood pressure as refractive hypotension is now believed to be the cause of death in victims. Although the effect of the whole extract on blood pressure has been studied, this is the first time the effect of Cleistanthin C.

Aim

To study the effect of Cleistanthin C on blood pressure of rats and the effectiveness of vasopressor drugs in hypotension if it occurs .

Objectives

1. To study the effect of intraperitoneal administration of Cleistanthin C on blood pressure of rats.
2. To study the effect of intravenous administration of Cleistanthin C on blood pressure of rats.
3. If hypotension occurs, to study the effect of administration of phenylephrine on the blood pressure of rats.

Review of literature

All substances are poisons; it is the dose that makes the poison (Paracelsus, sixteenth century toxicologist). This means any substance in the incorrect dose maybe a poison.

Poison is defined as toxicants that can cause death or illness even when taken in very small quantities.(1)

Toxicant is a substance that produces adverse biological effect.(1) A toxicant may be either physical or chemical in nature. The biological effect maybe chronic or acute.

Toxins, however, are specific proteins produced by living organisms.

Suicide is defined as the act of deliberately killing oneself .(2) The risk factor for which include various physical and mental illnesses.

Homicide is defined as injuries inflicted by another person with the intent to injure or kill.(3)

Suicide is one of the leading causes of death worldwide, with nearly 800,000 deaths globally attributed to it every year. (2) For every recorded case there are several attempted cases. Suicide is the second largest cause of death among the young population between the ages 15 to 29 years.(2) Ingestion of poisonous substances is among the most common method of suicide along with hanging and fire arms.

In India, the scenario is not very different. The incidence of suicide in India in 2013 was found to be 11.0 per 100000 population (4).In a 2010 survey, among the population of age 15 years or higher, the rate of suicide is about 3% which corresponds to a total of 187000 deaths among this age group. Men have a higher rate 26.3 per 100000 population as compared to 17.5 per 100000 population among

women. The majority of these cases were found to be in the age group of 15-29 years (56% women suicide, 40% men suicide). About half of the suicides in India are by poisoning.(5)

In the southern states of India(these include the states of Tamil Nadu ,Andhra Pradesh, Karnataka, Kerala, Puduchery, Andaman & Nicobar Islands and Lakshadweep) the situation is much grimmer. Here the suicide rate among men is 5.3% (as compared to the national average of 2.1%) and among women it is 4.5% (as compared to the national average of 2.0%). The rural population fares worse than the urban population. Here too the age group between 15-29 is at the maximum risk.(5) Tamil Nadu contributes to 12.5% of the total suicide burden of the country, the highest in the country.

Cleistanthus collinus:

Cleistanthus collinus is a shrub belonging to the family Euphorbiaceae. This family consists of about 140 species of plants. The species studied in this study is *Cleistanthus collinus*. This plant is found in most parts of India, some parts of Sri Lanka , Africa and Malaysia.(6) (7) .

Cleistanthus collinus is a short deciduous shrub. It bears oval leaves. The fruit is green initially but turns brown as it matures containing hard globular seed. The wood of this plant is strong, durable and resistant to termites. It is used for making wooden tools and instruments.

Pic.1:*Cleistanthus collinus* plant

All the parts of this shrub are considered poisonous. Leaves however are the most commonly used for both homicidal as well as suicidal purposes. This problem is

particularly evident in states of Tamil Nadu and Puducherry and is more common among women(8).

The mortality among the cases of *Cleistanthus collinus* poisoning maybe as high as 32% (8) . Other than for homicidal and suicidal purposes, this poisonous plant is also used as an abortifacient as well as cattle and fish poison. In traditional medicine, this plant has been used for treatment of various ailments such as gastrointestinal distress, headache and various skin ailments.

As it is found nearly everywhere in India, it is also known as *Garari* in Hindi, *Oduvanthalai* in Tamil, *Vadise* in Telugu*; Nilapala* in Malayalam, *Karada* in Oriya, *Badadarige* in Kannada and as *Karlajuri* in Bengali.

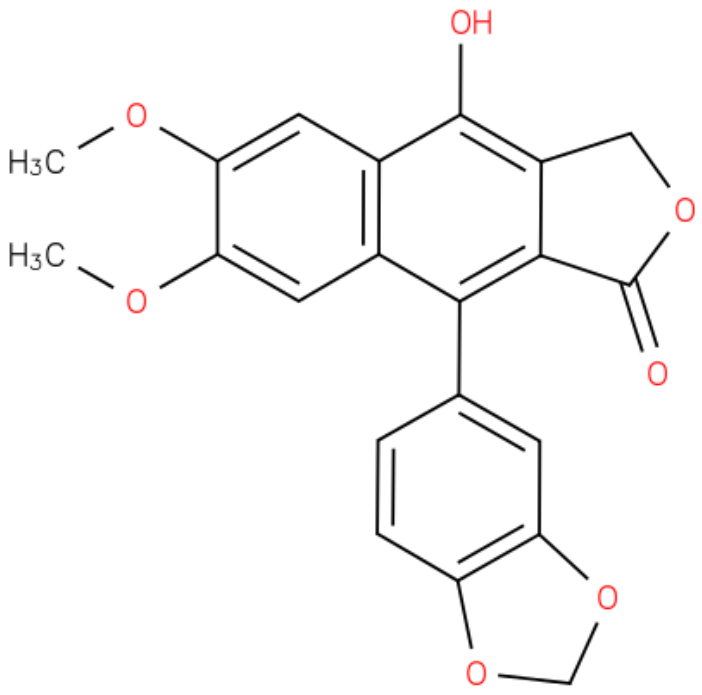
Phytochemistry of *Cleistanthus collinus* leaves:

The leaves of the *Cleistanthus collinus* plant show the presence of arylnapthalide lignans as well as their glycosides. They also show the presence of furofuranoid lignans.

There are a number of non toxic as well as toxic compounds present in the leaves of *Cleistanthus collinus* plant. Arylnapthalide lignans (both free and as glycosides) have been isolated.

Important compounds in the leaves include lignan lactones such as diphyllin **(Pic.2)**, collinusin, and Cleistanthins such as Cleistanthin A and Cleistanthin C .(9)

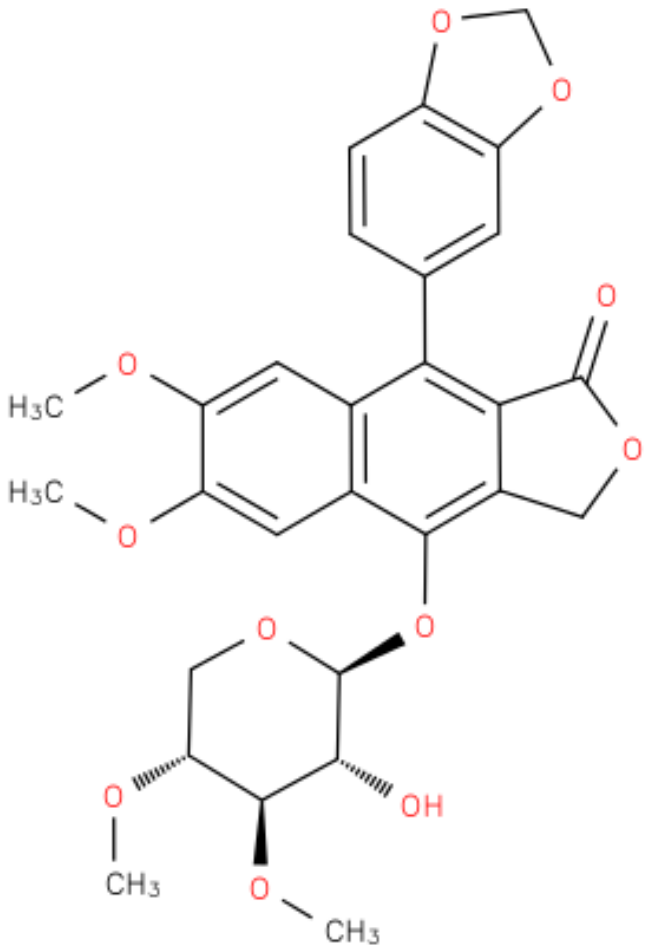
They also contain other chemicals such as β-sitosterol and Elagic acid.



Pic.2 : Chemical structure of Diphyllin (CHEBI:4645)

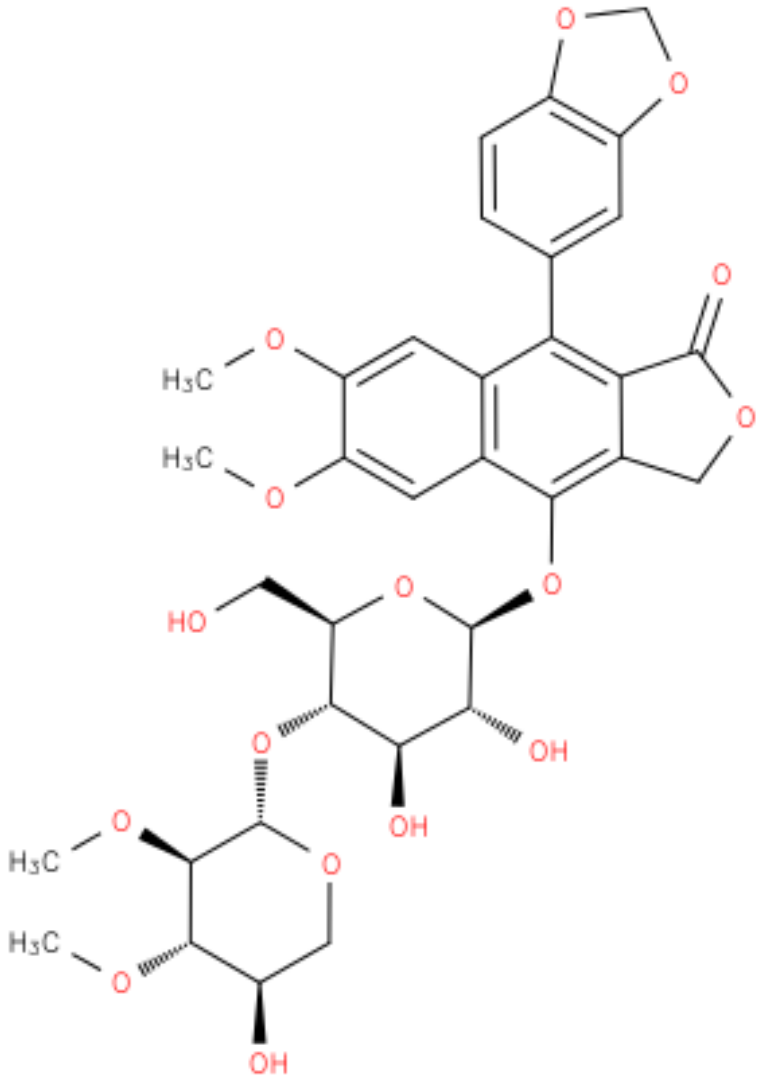
Cleistanthin A and Cleistanthin C have been identified as the 2 main toxic constituents of the *Cleistanthus collinus* plant. Both these compounds have been identified as glycosides of diphyllin.(10)

Cleistanthin A is the first Cleistanthin compound isolated in this plant. Chemically it is 4-*O*-3,4-di-*O*-methyl-β-D-xylopyranoside of 1,3-dihydronaphtho[2,3-*c*]furan-4-ol which is substituted by an oxo group at position 1, methoxy groups at positions 6 and 7, and a 1,3-benzodioxol-5-yl group at position 9.(11) (Pic.3)



Pic.3: Structure of Cleistanthin A(CHEBI:3737)

In this dissertation, Cleistanthin C is the compound of interest. It is cleistanthin A in which the hydroxy group at position 4 of the glucoside moiety has been converted to the corresponding 2,3-di-*O*-methyl-β-D-xylopyranoside.(12) **(Pic.4)**



Pic.4: Structure of Cleistanthin C (CHEBI:84408)

***Cleistanthus collinus* poisoning**:

The cases of *Cleistanthus collinus* poisoning are mainly from rural areas. It is more common among women. It is the availability and free access to the leaves of these plants that makes it such a common method of suicide in these parts.

There are mainly 2 ways in which the leaves of this plant are consumed.

- The leaves are either crushed into a paste and consumed directly, or
- The leaves are boiled in water and the victim drinks the resulting concoction.(13).

The boiled decoction of the leaves are believed to be more toxic than the crushed leaves (13). The reason may probably be that while boiling more leaves maybe used while it may not be very palatable to consume large amounts of the leaves after grinding them.

The patients are brought to the hospital with an array of signs and symptoms which may be range from asymptomatic to non specific symptoms to death. Therefore a careful history is very important to zero onto the correct history of poisoning.

The time between the consumption of the leaves and the initiation of treatment, the actual amount of leaves consumed (which the victim describes in number of handfuls of leaves) and the mode of consumption of the leaves are crucial determinants of prognosis.

Clinical presentation: (13)

Symptoms: Patients are usually brought with the following symptoms:

1. Abdominal pain
2. Vomiting
3. Nausea
4. Diarrhoea
5. Blurred vision
6. Cramps
7. Collapse
8. Death

Signs: Examination of these patients reveals the following:

1. Respiratory failure and Hypoxia
2. Hypokalemia
3. Metabolic acidosis
4. Hyperchloremic high anion gap acidosis (Type I DRTA)
5. Alkaline urine
6. Leucocytosis
7. Cardiac arrythmias – QTc prolongation and ST-T wave changes
8. Elevated liver and cardiac enzymes
9. ARDS
10. Intractable hypotension and distributive shock
11. Coagulopathy

Treatment:

There is no specific antidote available for this poisoning, hence the treatment is mainly symptomatic. These include:

1. Intravenous fluids mainly crystalloids.
2. Potassium supplements including parenteral potassium chloride.
3. Correction of acidosis with bicarbonate supplements.
4. Oxygen and assisted ventilation.
5. Cardiac Pacing
6. Ionotropes
7. N-acetyl cystine

Some victims undergo an asymptomatic phase of 1-4 days followed by rapid deterioration of the condition. Death typically occurs by the 3^{rd} to 7^{th} day.(14)

Even after more than 3 decades of research, the mechanism of action of the poison or the cause of death in this poisoning has not yet been fully understood.

Different hypotheses have been suggested regarding the cause of death in *Cleistanthus collinus* poisoning. These include renal failure, ARDS and respiratory arrest, cardiac arrhythmias and shock.(8) (15) (16).

Myasthenic crisis like syndrome has also been reported.(17)

Distributive shock due to inappropriate vasodilatation has been associated with high mortality and therefore considered to evaluate in this study.

Mechanism of action:

There have been various mechanisms of action proposed for the effects of *Cleistanthus collinus* poisoning. They are

- Potassium channel blockade
- ATP and glutathione depletion
- Neuromuscular blockade
- Sodium potassium pump inhibition
- Neutrophilic granulocytosis
- DNA synthesis blockade
- Cytotoxicity
- Break in DNA
- Inhibition of LDH isoenzymes.

All these effects have been demonstrated in various clinical and animal studies. Some important studies are mentioned below.

Animal studies:

1. **Rao *et al* (1970):** Demonstrated neurtophilic granulocytosis in Swiss mice, albino rats, mongrel cats and rhesus monkeys in response to Cleistanthin extract.(18)

2. **Nandakumar *et al* (1989):** Demonstrated weakness of diaphragmatic muscles by reduction in miniature end plate potential and respiratory failure and cramps in mice. (19)

3. **Sarathchandra *et al* (1997) :** Showed the reduction in lactate dehydrogenase and ATPase depletion in various tissues in rabbits and rats after administration of boiled extract of *Cleistanthus collinus* leaves.(20)

4. **Maneksh *et al* (2010):** Demonstrated type I distal renal tubular acidosis and type II respiratory failure in rats.(21)

5. **Kumar *et al* (2010):** Demonstrated a probable alpha adrenergic receptor blocking property of the whole extract of Cleistanthus collinus on various isolated smooth muscle preparations. (22)

6. **Kettimuthu *et al* (2011)**: Demonstrated the inhibition of vacuolar ATPase in renal brush border membrane and basolateral membrane in rat kidneys. (23)

Clinical studies:

1. **Thomas *et al* (1987):** published case reports of 32 victims of this poisoning of which 9 people had died, 8 of which due to cardiac arrest. The surviving patients also showed hypokalemia.(13)

2. **Thomas *et al* (1991):** published 11 cases explaining the reason for hypokalemia as renal tubular potassium leak leading to heavy loss of potassium in the urine.(14)

3. **Benjamin *et al* (2006):** published a case report where the victim was a 24 year old man. It was here, that acute respiratory distress syndrome, renal tubular acidosis and distributive shock were first reported.(24)

4. **Damodaram *et al* (2008):** reported Mysthenic crisis like syndrome in one of the victims.(17)

5. **Nampoothiri *et al* (2010):** in a prospective study on 32 patients ,demonstrated the presence of type I DRTA.(15)

The wide spectrum of clinical features makes determining the cause of death very difficult.

Various cell line studies have suggested cytotoxcicity with *Cleistanthus collinus*.(25)

Anti cancer properties of *Cleistanthus collinus* has also been seen against human epidermal nasopharyngeal carcinoma.(26)

Phenylephrine induced vasodilatation

Phenylephrine is a selective alpha I agonist, used as a vasoconstrictor and a mydriatic. Being a vasoconstrictor, it is sometimes used for treatment of shock.

while testing the vasoconstrictor action of phenyl ephrine in *C.collinus* setting in the spiral strips of isolated goat artery ,we found that Cleistanthin C per se did not have any effect on the vascular tone of the goat arteries, however addition of sufficient dose of phenylephrine led to a rapid and dramatic decrease in the tone of the vessel.(Unpublished work)

This led us to explore the possibility of phenylephrine induced vasodilatation. In a series of experiments it was discovered that phenylephrine, in high nitric oxide environment can actually produce vasodilatation instead of vasoconstriction.(27)

Rationale behind the study:

Distributive shock secondary to inappropriate vasodilatation has consistently been recorded in most cases of fatal cases of Cleistanthin C poisoning. This makes the effect of Cleistanthins on blood pressure a prime area for research.

Although effect of Cleistanthin compounds, both as whole extract as well as individual compounds have been studied , to the best of our knowledge, effect of alpha agonist like Phenylephrine on hypotension produced by Cleistanthin C has never been demonstrated *in vivo*.

The idea here is to demonstrate that alpha agonists are not only effective in the treatment of distributive shock produced by *Cleistanthus collinus* poisoning but in fact detrimental to the patients.

Materials and Methods

Animal model (wistar rat) was used to study the effects of Cleistanthin C on blood pressure . This study was done over a period of one and half years.

Christian medical college, Vellore, Institutional review board approval was obtained prior to commencement of the study (IRB No.9337, Dated- 3.3.2015). Approval of the Institutional Animal Ethics Committee, Christian Medical College, Vellore was also obtained.

The study consists of 2 parts:

- Isolation of Cleistanthin C
- In vivo experiments on Wistar rats

ISOLATION OF CLEISTANTHIN –C:

MATERIALS REQUIRED:

- Fresh leaves of *Cleistanthus collinus*
- Distilled water
- Weighing scale
- Glass beaker – 5 litre capacity
- Electric hot plate
- Separation funnel
- Filter paper/cotton
- Glass plates for Thin Layer Chromatography (TLC)
- Silica gel (for TLC)
- TLC tanks (made of thick glass)
- Solvents
  - n – Heptane
  - Chloroform
  - Ethanol
  - Methanol

METHOD:

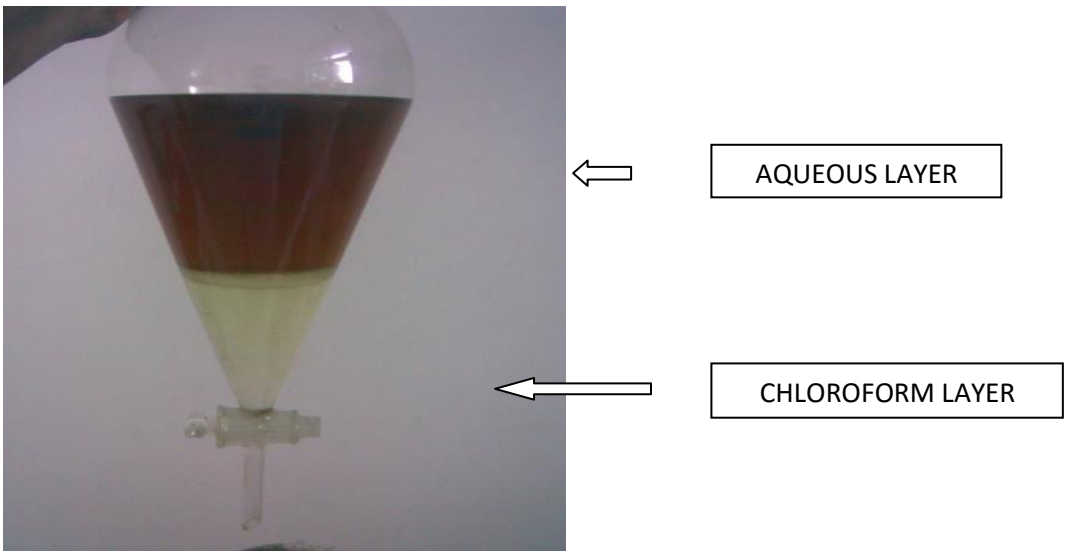
Preparation of fresh leaf boiled extract of *C.collinus*

100 grams of fresh leaves of *Cleistanthus collinus* was weighed washed and put in a beaker containing 3 litres of distilled water. The leaves were then boiled for 30 minutes **(Pic.5)**. The water which had turned dark by now was then strained out and the leaves were discarded.

Pic. 5: Boiling the fresh leaves of *Cleistanthus collinus*

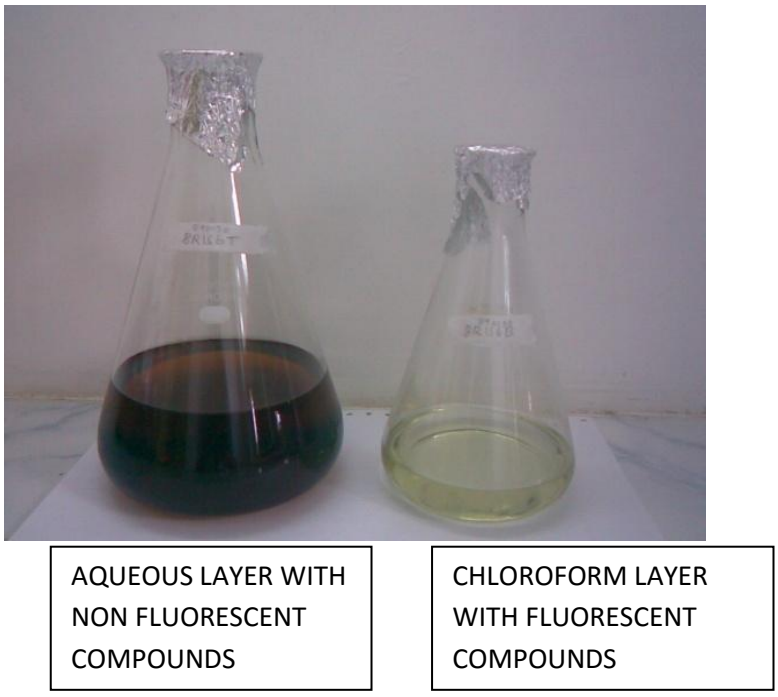
Equal volume of chloroform was added and mixed vigorously. Mixture was allowed to stand overnight. Next day the mixture is transferred to a separation funnel and allowed to stand for 15-20 minutes. A clear separation was visible between water and chloroform with water above and chloroform below **(Liquid-liquid partition) (Pic.6)**.

The fluorescent compounds present in the leaf extract were highly soluble in chloroform and goes into the chloroform layer **(Pic.7)**.



Pic.6: Liquid-liquid partition

The tap of the funnel was then opened and the chloroform layer was collected. This chloroform with the fluorescent compounds was concentrated using a rotary evaporator **(Pic.8)** and most of the chloroform was recovered for reuse.

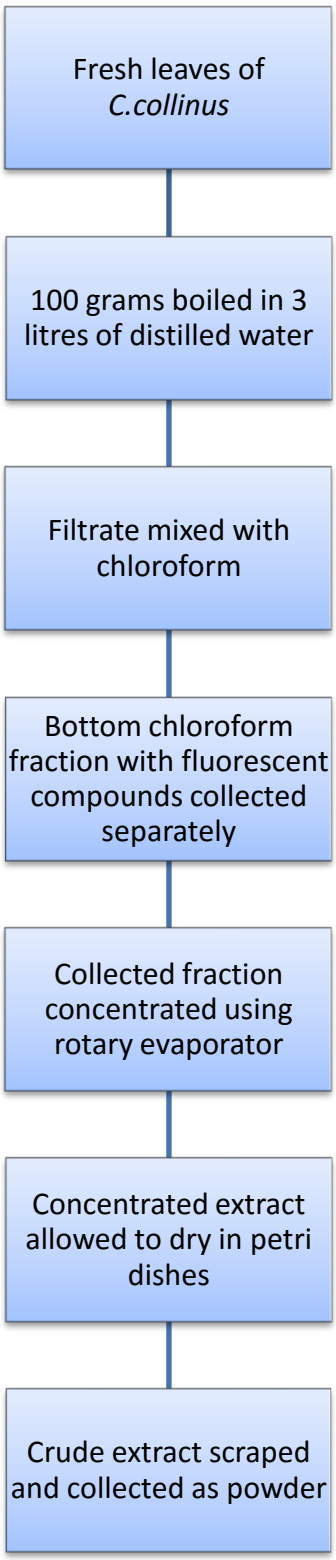


Pic.7: Products of liquid-liquid partitioning

The concentrated extract was poured into petri dishes and allowed to dry. Once dry, the petri dishes will have a greenish layer of fluorescent compounds at the bottom. This layer was scraped off and collected in the form of a powder and weighed. We refer to this powder as **crude extract (Pic.9).**

Pic.8: Rotary evaporator used to concentrate the chloroform fraction

Pic. 9: Crude extract of *C.collinus* leaves



Pic.10 : Flow chart depicting preparation of crude extract of *C.collinus*.

Thin layer chromatography:

Preparation of thin layer chromatography (TLC) plates:

35 mg of silica gel powder was weighed. This powder was then mixed with 75ml of distilled water to prepare a mixture called slurry. A special spreader was used to coat square glass plates (10cm×10cm) with this slurry. The spreader ensures that the thickness of the slurry film on the glass plate was 0.5mm uniformly **(Pic.11)**. Care must be taken to ensure that the glass plates were free of dirt and grease. It must be wiped with acetone and dried before the application of the silica gel slurry.

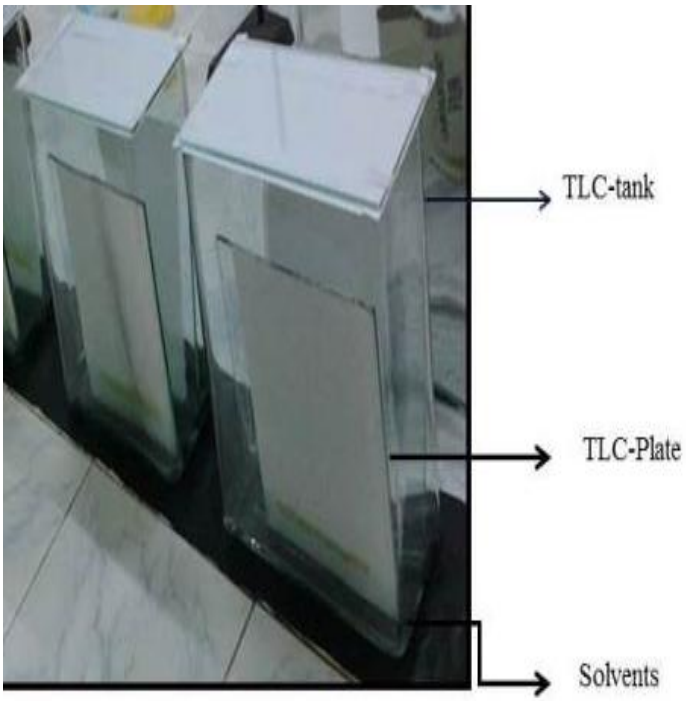
Pic.11: Glass plates covered in silica gel with spreader

The coated plates were then air dried till all the moisture evaporates. The plates were kept in a hot air oven for 1 hour just prior to running the thin layer chromatography (TLC) to remove any moisture which may have been absorbed by the silica gel. This process is known as activation of the plates.

Running the thin layer chromatography:

100mg of the crude extract was dissolved in 2ml of chloroform. This was applied as a single horizontal lines (spotted) 1-1.5cm from the base of the each plate and allowed to dry. A single spot of reference solution was also applied to recognize the compare band later in the process. This was the fixed phase of thin layer chromatography.

The mobile phase was prepared by mixing 3 solvents namely n-heptane, chloroform and absolute ethanol in a ratio of 5:5:1. Tanks made of thick glass were taken and about 100ml of the mobile phase was poured into each tank.

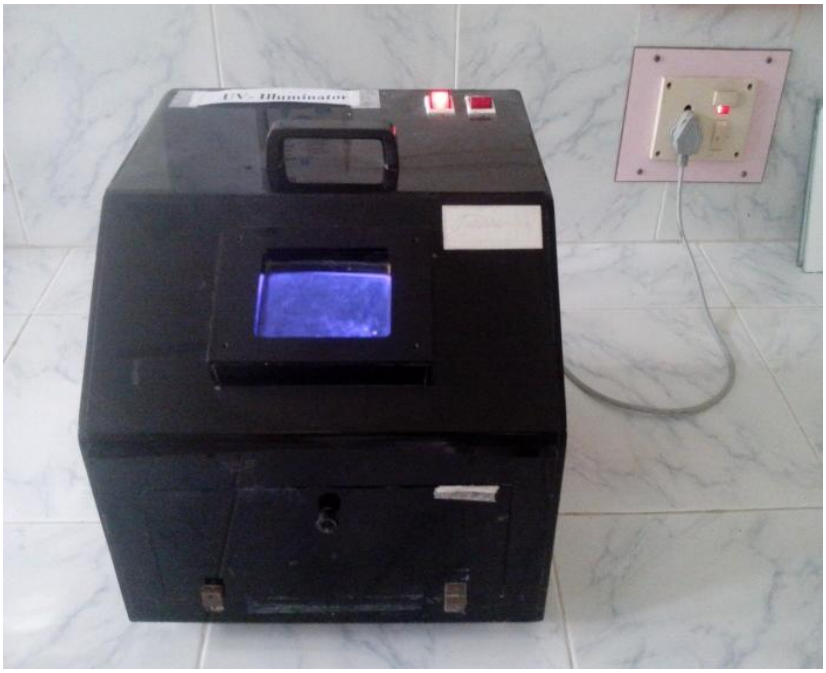


Pic.12: TLC tanks with the crude extract for seperation

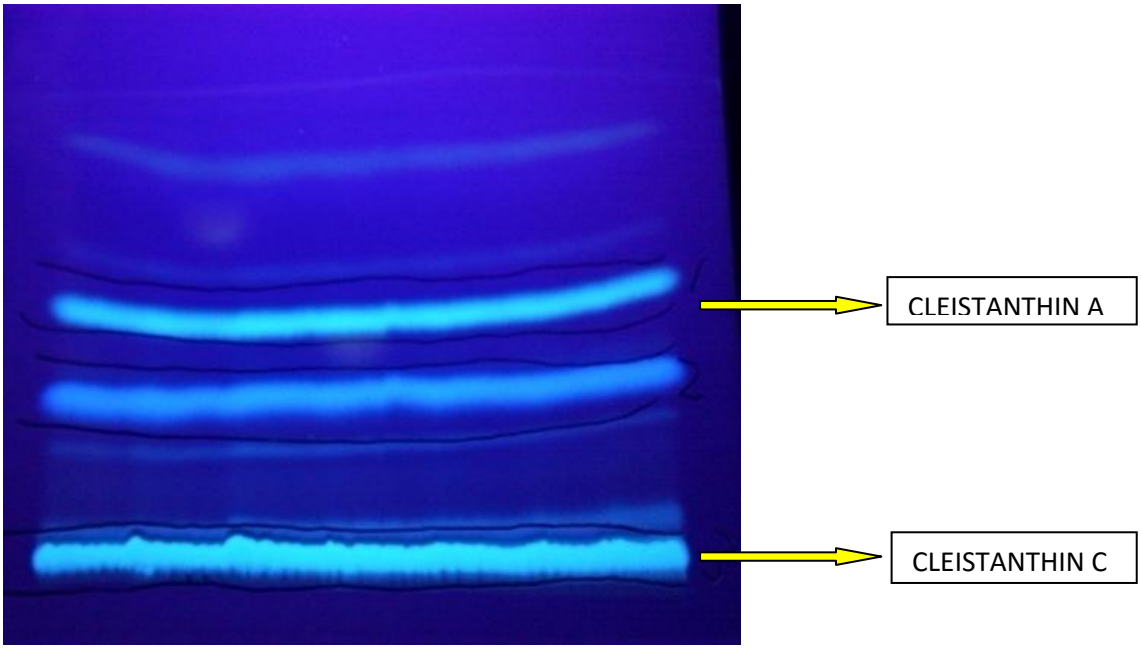
The base of the spotted thin layer chromatography plates were immersed into the mobile phase taking care that the spotted area does not come in direct contact with the mobile phase. The tanks were covered to minimize the evaporation of the mobile phase as it may alter the ratio of the solvents in it, since different solvents evaporate at different rate **(Pic.12)**.

The mobile phase moves up the silica gel layer by capillary action separating the crude fraction into bands of individual compounds. The process was run till the mobile phase reaches the top of the plate. The plates were then removed from the tank and allowed to air dry.

The plates were observed under ultraviolet lamp **(Pic.13)** and the band containing the required compound (here Cleistanthin C) was recognised with the help of the reference spot. It was marked with the help of a probe **(Pic.14).**



Pic.13: U-V Box



Pic.14: TLC plate showing bands of different fractions found in the crude extract under UV light.

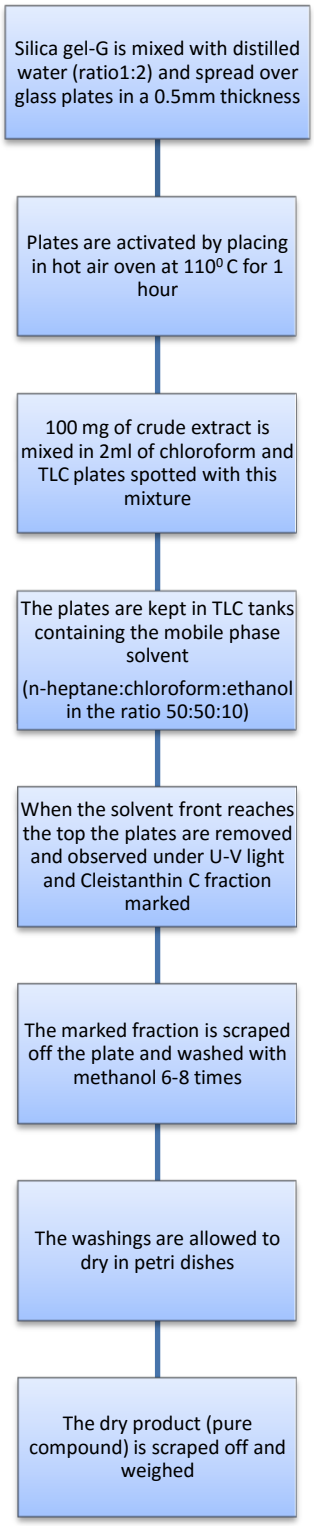
The bands containing the compound was now scraped off from all the plates and collected. It was mixed with about 50ml of methanol and agitated vigorously. The mixture was allowed to stand undisturbed for a few hours to allow silica gel particles to settle down. The compound dissolves in the methanol.

The supernatant methanol was then collected in a 50 ml centrifuge tube. The tube was centrifuged at 2400 rpm for 10 minutes to remove any remnants of the silica gel.

The supernatant was transferred into a wide Petri dish and the methanol was allowed to evaporate till dry. What remains in the dish was the compound which was then scraped off using a clean surgical blade, collected and weighed.

A sample of the compound was taken and thin layer chromatography was done and observed under ultraviolet light to ensure that there were no contaminants.

In case of contamination, thin layer chromatography was repeated on the product compound till a compound of sufficient purity was obtained.



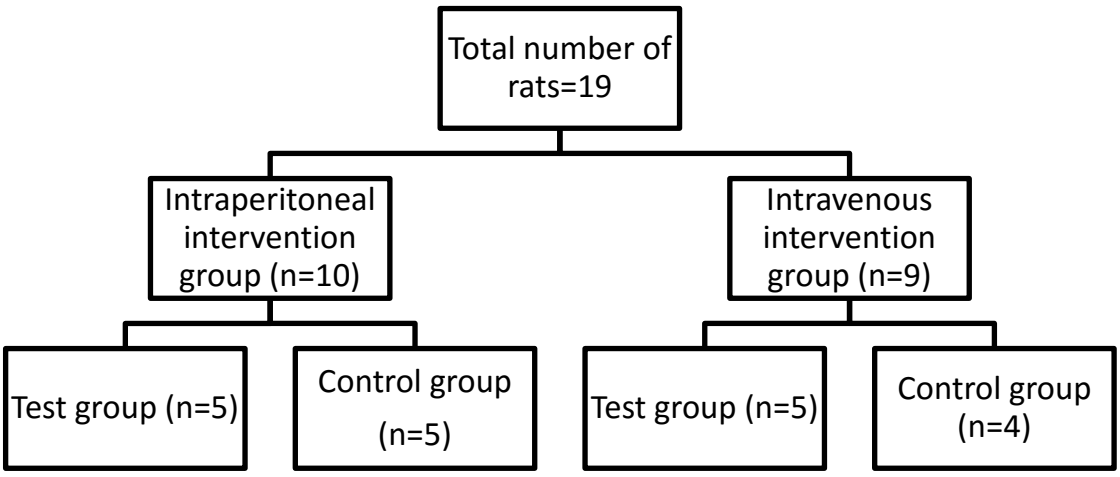
Pic .15: Flow chart depicting the procedure to obtain purified Cleistanthin C from crude extract by Thin layer Chromatography.

ANIMAL EXPERIMENTS: A total of 19 wistar rats (150-350 gm, both sexes) were used for the experiments. Animals were divided into 2groups:

a) Intraperitoneal intervention group (n=10)

b) Intravenous intervention group (n=9)

Intraperitoneal group was further subdivided into a test group (n=5) and a control group (n=5). The intravenous group had 5 animals in the test group and 4 animals in the control group. Either left or the right carotid artery was cannulated in all the rats for recording the arterial blood pressure.



Carotid artery cannulation:

Materials required:

- Inj. Ketamine
- Inj. Xylazine
- Inj. Heparin
- Normal saline
- 24G IV cannula
- 3-way stop cork
- 1cc syringe
- Surgical instruments (scissors, forceps, spatula)
- Thread
- Pressure transducer
- CMC Data acquisition system
- Laptop

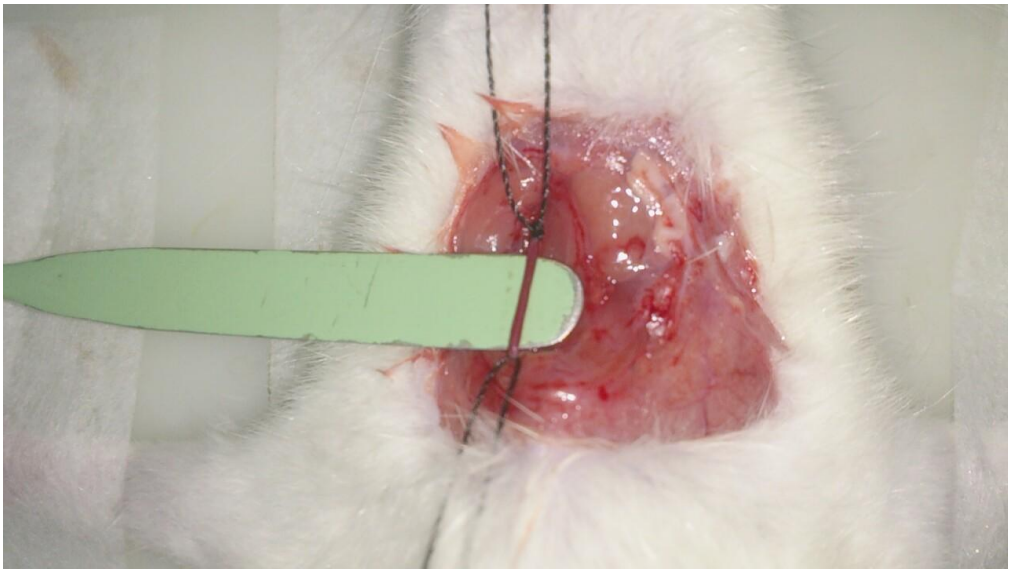
Procedure

Wistar rats were anesthetised with ketamine (10mg/100gm BW) and xylazine intraperitoneally **(Pic.16)**. A 100 IU/ml stock solution of heparinised saline was made (250µl heparin in 10ml ml normal saline). A 24G cannula, a 3-way stop cork and a pressure transducer were heparinised with the stock solution and kept ready. Once under anaesthesia, the rat was placed supine on a dissection board and the limbs were secured with the help of adhesive plasters.

Pic.16: Anaesthesia being administered intraperitoneally.

The skin over the ventral side of the neck was incised and separated from the underlying subcutaneous tissue. The sub maxillary glands were retracted. Being highly vascular, it was not removed to prevent blood loss.

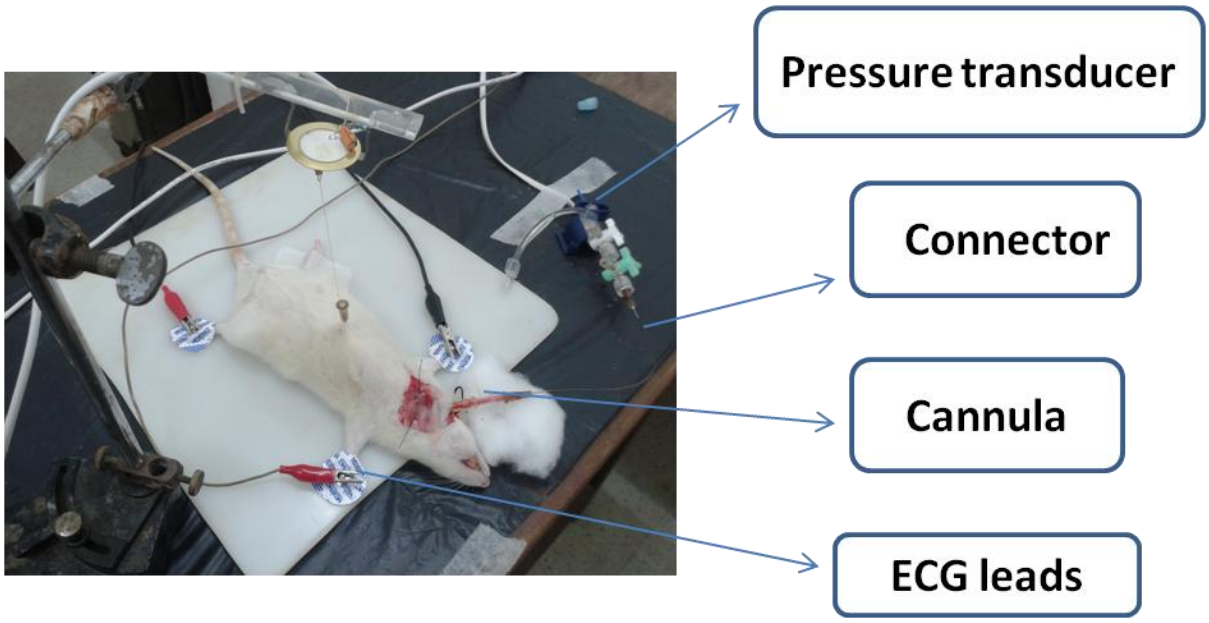
The neck muscles, namely, sternomastoid and sternohyoid were separated and retracted. This exposes the omohyoid muscle which was either retracted or excised. The trachea now becomes visible. The carotid sheath, which is seen just lateral to the trachea was identified with the help of the pulsations of the carotid artery. The carotid sheath was opened with the help of forceps and the carotid artery was carefully separated from the vagus nerve and isolated. Care was taken that the artery was free of any remnants of the carotid sheath as the tough tissues in the latter may make cannulation difficult. The distal part of the exposed artery was ligated. A loose tie was applied at the proximal end to secure the cannula after cannulation. A spatula was inserted below the carotid artery to provide a firm base for cannulation **(Pic.17)**.



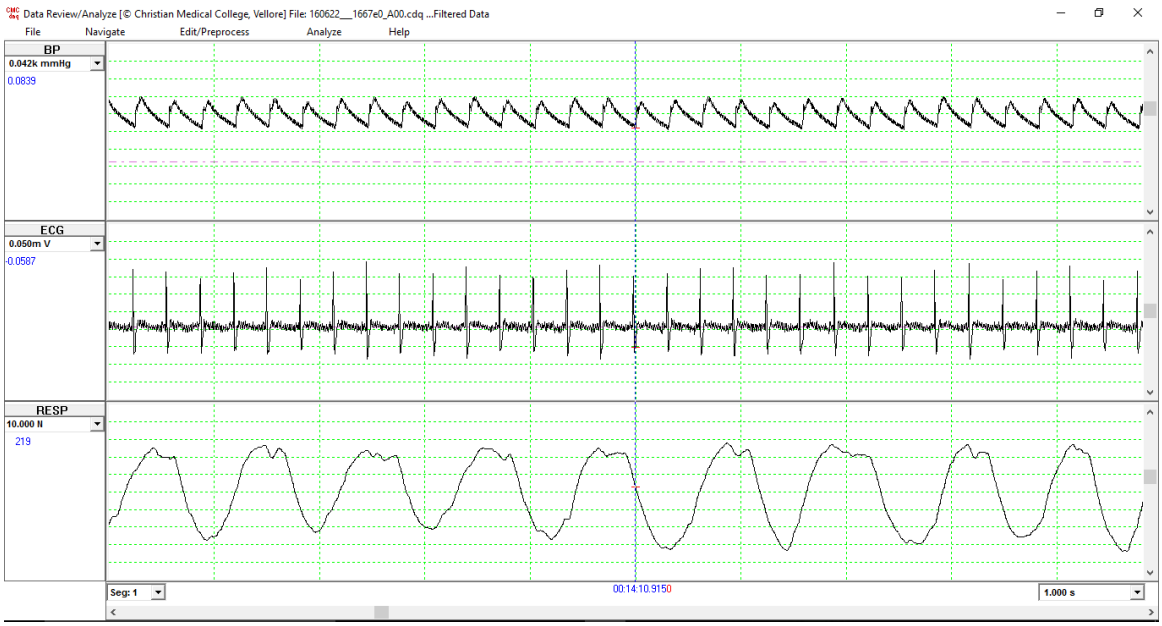
Pic.17: Right Carotid isolated for cannulation

The heparinised 24G cannula was used to cannulate the artery in the distal to proximal direction. Once the cannula was in the artery, the stillet was removed and the cannula was connected to the pressure transducer via a pre heparinised 3-way stop cork (Pic. 18). The cannula was then flushed with heparinised saline to prevent any blockage to the cannula due to clot and to keep it patent.

The pressure transducer was now connected to CMC data acquisition system (CMC Daq) which was further connected to a laptop. The raw data was recorded on the laptop. The data was analysed using Igor Pro and LabChart 8 Reader software.



Pic.18: Post Cannulation



Pic.19: Screenshot of an experiment on CMC Daq

Intraperitoneal administration of Cleistanthin C

Materials:

- **Wistar rats**
- **Cleistanthin C**
- **30% ethanol**
- **Inj. Phenylephrine**
- **Normal saline**

Method :

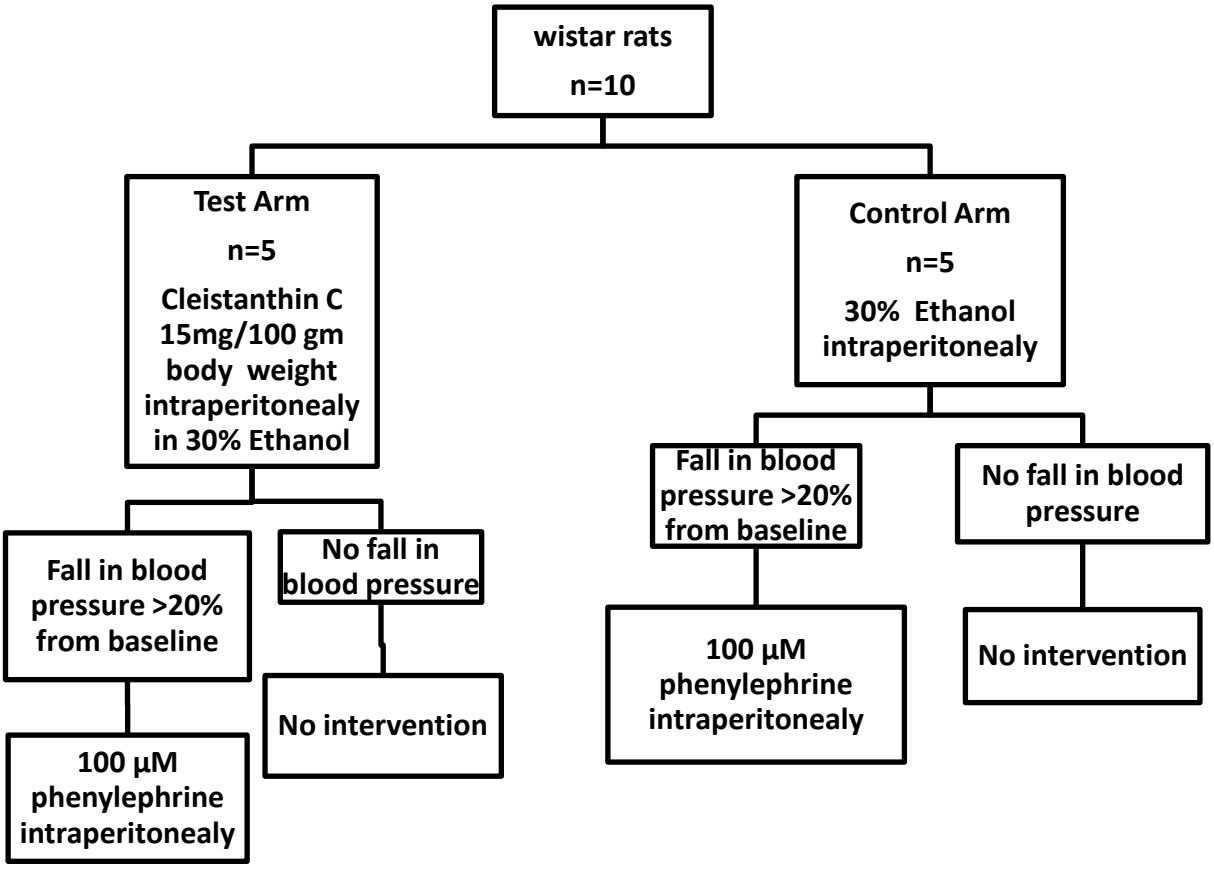
The rats were divided into 2 groups: Test group (n=5) and Control group (n=5) and the carotid artery was cannulated as described above. The rats were left to stabilise for 15 minutes. The test rats were administered Cleistanthin C at a dose of 15mg/100 gm body weight, dissolved in 600µl of 30% ethanol, intraperitoneally.

The pressure waves were recorded over 8 hours or the death of the rat whichever was earlier. In case of fall in blood pressure >20% from the baseline 100µM injection Phenylephrine was to be administered intraperitoneally and the effect observed. (However, we did not observe an adequate drop in the blood pressure during the intraperitoneal administration of Cleistanthin C, hence, we did not administer Phenylephrine)

In the control group, the rats were administered 600µl of 30% ethanol intraperitoneally and the pressure recording was done over 8 hours.

In both the groups, adequate volume replacement was done periodically by administering normal saline intraperitoneally.

Study design for intraperitoneal intervention



Intravenous administration of Cleistanthin C

Materials:

- **Wistar rats**
- **Cleistanthin C**
- **30% ethanol**
- **Normal saline**
- **IV cannula 24G**
- **IV perfusion set**
- **Inj. Phenylephrine.**

To observe the effect of Cleistanthin C on blood pressure:

In this arm of the experiment, the rats were divided into test (n=5) and control (n=4) groups. After carotid artery cannulation, the rats in both groups were also cannulated through one of the femoral vein.

For this a small incision was made over the ventral aspect of either left or the right thigh. The femoral vein was identified and isolated by cleaning the tissue around it.

The vein was cannulated using a 24G cannula which was secured in place with the help of a thread. After removing the stillet, the cannula was flushed with heparinised normal saline to keep the cannula patent. The cannula was then connected to a 3 way tap through which the drugs as well as fluids were infused.

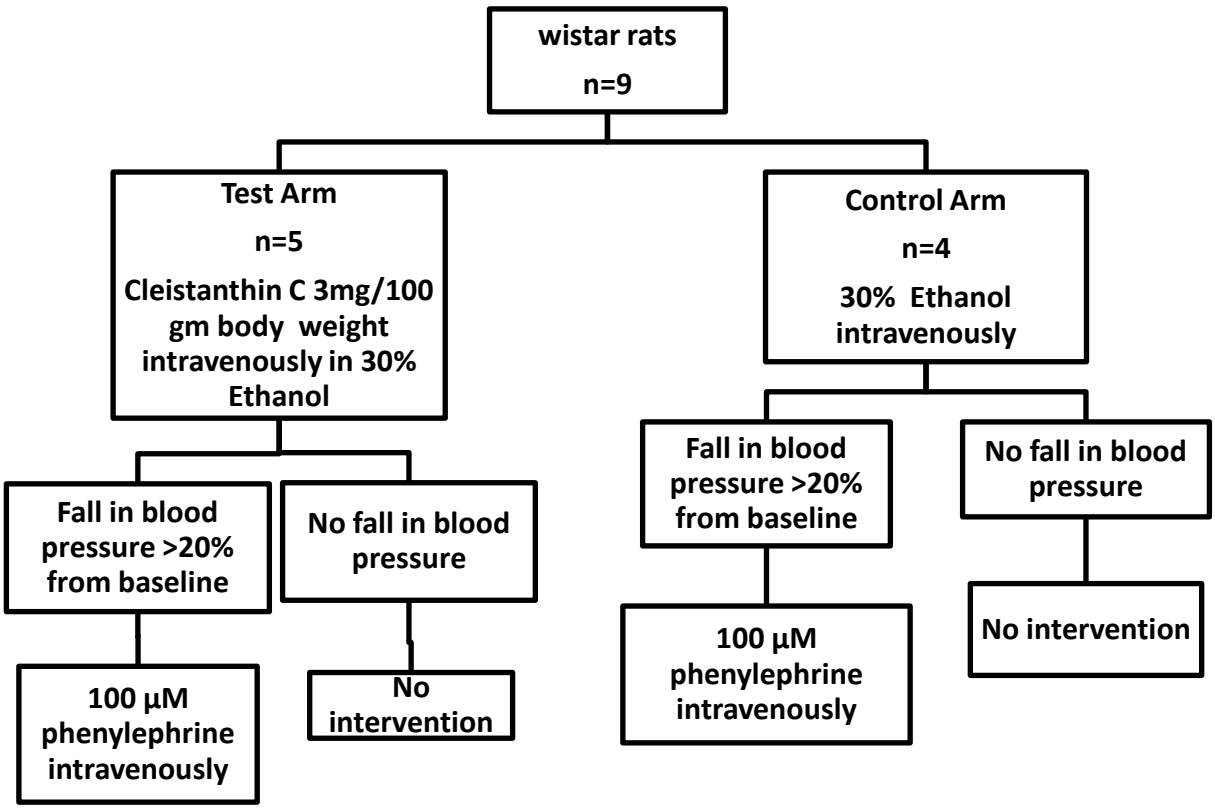
Continuous fluid perfusion was maintained with intravenous normal saline at the rate of 10 ml/kg/hour.

The test rats were administered Cleistanthin C at a dose of 3mg/100 gm body weight, (lethal dose determined in our previous studies) dissolved in 600µl of 30% ethanol, intravenously. The pressure waves were recorded over 8 hours or the death of the animal, whichever was earlier.

In case of fall in blood pressure >20% from the baseline 100µM injection Phenylephrine was administered intravenously and the effect observed.

In the control group, the rats were administered 600µl of ethanol intraperitoneally and the pressure recording was done over 8 hours or the death of the rat, whichever was earlier.

Study design for Intravenous intervention



DATA ANALYSIS:

The raw data was converted to text file which was loaded into LabChart 8 Reader (AD instruments). Pressure- time curve was generated on Igor Pro and LabChart 8 Reader (AD instruments) and 3 points were selected for analysis:

1. Baseline – Before intervention after the blood pressure had stabilized.
2. Post intervention- At the point of maximum change in blood pressure.
3. Terminal event- At the end of 8 hours or just before the death of the rat.

The parameters analysed were:

1. Heart rate
2. Systolic blood pressure
3. Diastolic blood pressure
4. Pulse pressure
5. Mean arterial pressure.

At each point the value is averaged over 1 minute.

Percentage change of mean arterial pressure from baseline was also compared.

STATISTICAL ANALYSIS:

All values are expressed as Mean ± SD.

Tests for significance were calculated using Wilcoxon- Signed- Rank test for values within the same group.

Mann Whitney U test was used to test significance between the test and control groups.

A p-value of <0.05 was considered significant.

The statistical analysis was done using SPSS and PSPP software.

Results

In the Intraperitoneal intervention group, both the test(n=5) as well as control(n=5) animals stayed alive at the end of 8 hours even at15mg/100 gm body weight, which is 5 times the lethal dose (LD_{100}) previously determined in our laboratory. This led us to change the route of administration to intravenous.

In the intravenous intervention group, there was **100% mortality** in the test group (n=5) even at 3mg/100gm body weight which the usual lethal dose (LD_{100}) determined in our laboratory (Dr. Neetu Prince, MD thesis) (Manuscript under review for publication), whereas all the animals survived in the control group (n=4).

Intravenous injection of Cleistanthin C caused a drop in blood pressure. Administration of Phenylephrine either did not cause an increase or caused a small increase followed by a precipitous drop in the blood pressure leading to the death of the animal.

In the control group of animals however, the blood pressure remained fairly stable throughout the 8 hours.

INTRAPERITONEAL INTERVENTION GROUP:

The figures below show the representative pressure traces of test (fig.1) and control (fig.2) groups.

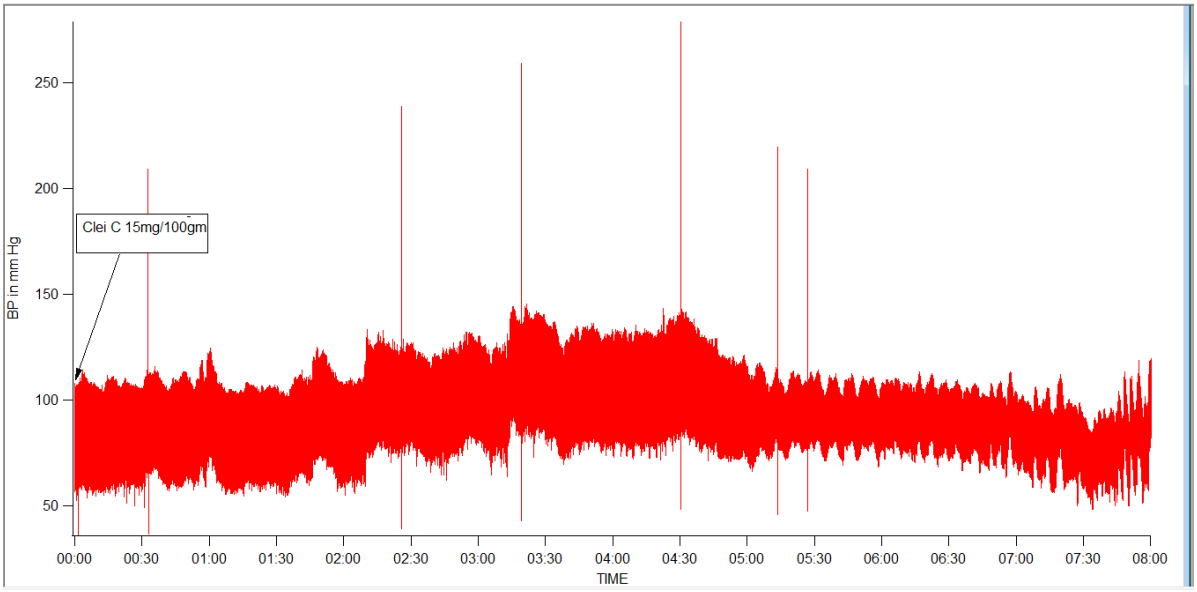


Fig.1: Effect of Intraperitoneal administration of Cleistanthin C on blood pressure of rats.

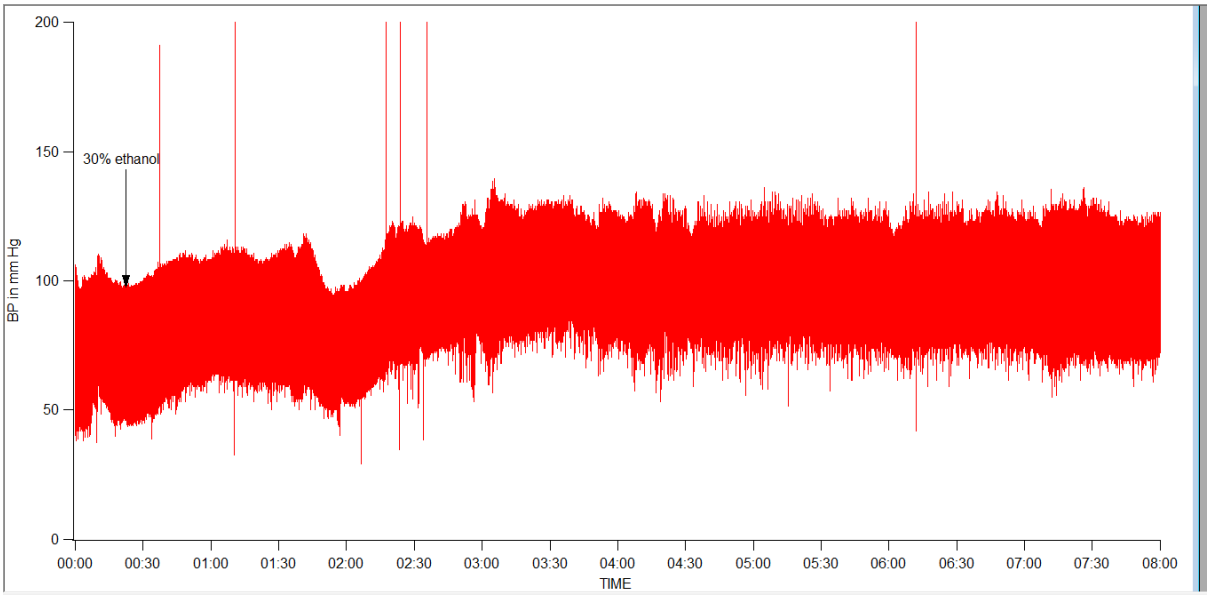


Fig.2: Effect of Intraperitoneal injection of 30% ethanol on blood pressure of rats.

Effect of intraperitoneal administration of Cleistanthin C on various hemodynamic parameters:

			INTRAPERITONEAL INTERVENTION			
PARAMETERS	BASELINE	TEST POST CLEISTANTHIN C	END OF EXPERIMENT	BASELINE	CONTROL POST ETHANOL	END OF EXPERIMENT
HEART RATE(BPM)	197±65.9	222±64	219±34.6	203±52.2	117±34.5	173±48.7
SYSTOLIC BP(mm Hg)	98.4±12.7	114±13.1	90.4±25.9	92.6±15.3	115±10.5	99.8±15.2
DIASTOLIC BP(mm Hg)	66.2±9.7	78.6±6.7	60.4±17.2	55±12.2	75.2±13.1	57.4±18.3
PULSE PRESSURE (mm Hg)	32.2±8	35.2±8.4	30±13.9	37.6±4.4	39.6±4.2	43±3.8
MEAN ARTERIAL PRESSURE(mm Hg)	78.4±10.7	91.6±7.6	70.2±17.6	67.6±13.2	88.4±12.1	71.4±17.5
% CHANGE IN MAP	0±0	18.78±14.2	-9±24.2	0±0	30.7±19.4	5.8±20.1

Table 1: The above table shows the effect of intraperitoneal injection of Cleistanthin C on various hemodynamic parameters. These include: 1.Heart Rate, 2. Systolic blood pressure, 3. Diastolic blood pressure, 4. Pulse pressure, 5. Mean arterial pressure and 6. Percentage change in mean arterial pressure.

EFFECT ON MEAN ARTERIAL PRESSURE:

The following graphs show the effect of intraperitoneal injection of Cleistanthin C on Mean Arterial Pressure (MAP) (Fig.3) as compared to that in control animals (Fig.4)

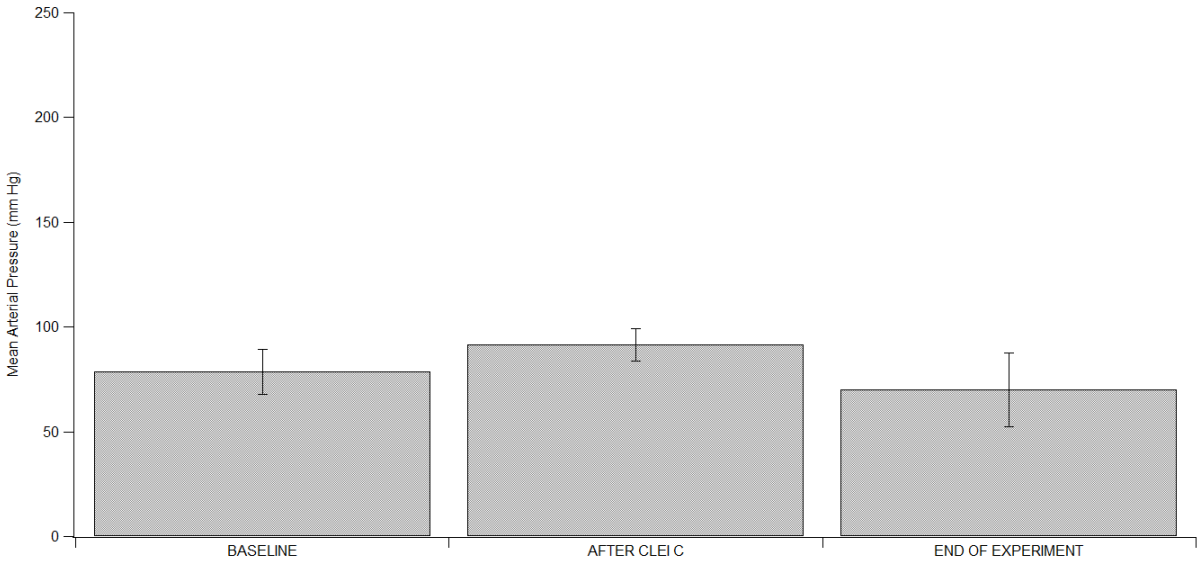


Fig. 3: Effect of Intraperitoneal injection of Cleistanthin C on mean arterial pressure of rats. MAP increased from 78.4±10.7mm Hg to 91.6± 7.6 mmHg (p=0.068) before dropping to 70.2±17.6 mmHg (p=0.223) at the end of 8 hours.

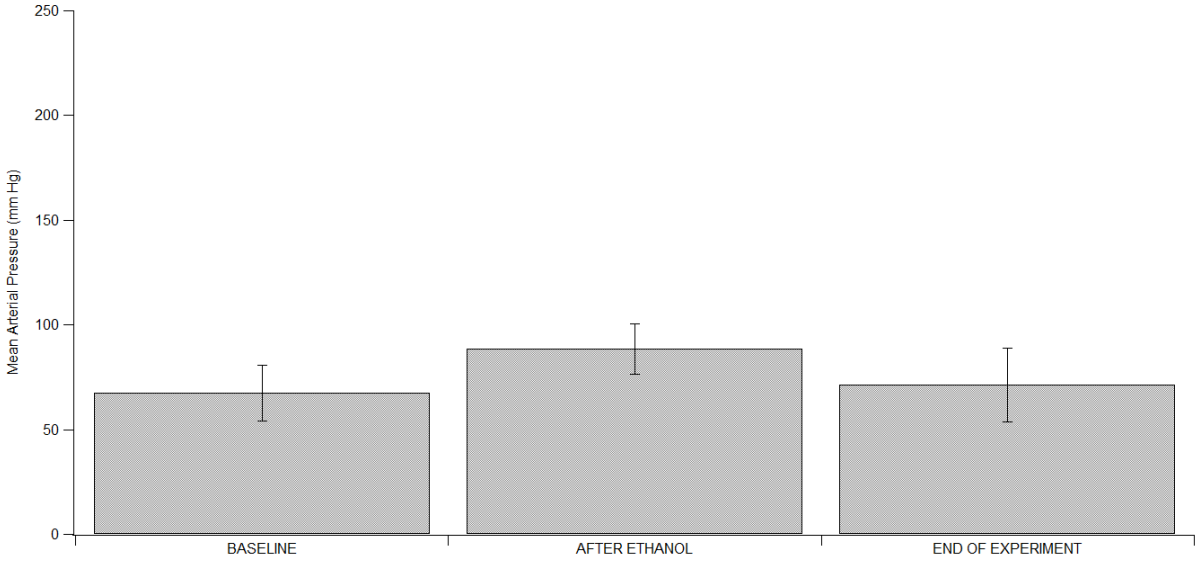


Fig. 4: Effect of Intraperitoneal injection of 30% ethanol on mean arterial pressure of rats. MAP increased from 67.6±13.2mm Hg to 88.4± 12.1mmHg (p=0.043)* before dropping to 71.4±17.5 mmHg (p=0.5) at the end of 8 hours.

PERCENTAGE CHANGE IN MEAN ARTERIAL PRESSURE:

The following graphs show percentage change in on Mean Arterial Pressure (MAP) after intraperitoneal injection of Cleistanthin C (Fig.5) as compared to that in control animals (Fig.6)

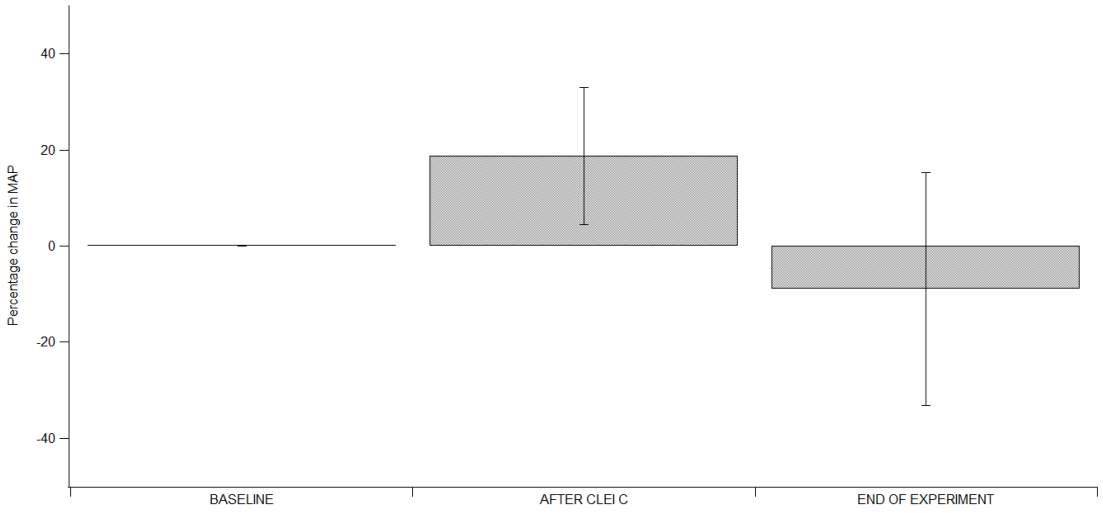


Fig.5: Shows the percentage change in mean arterial pressure test group. There was a percentage increase in blood pressure of 18.78±14.2 (p=0.043)* in the test group after administration of Cleistanthin C but there was drop of 9±24.2 percent (p=0.225) from the baseline at the end of 8 hours.

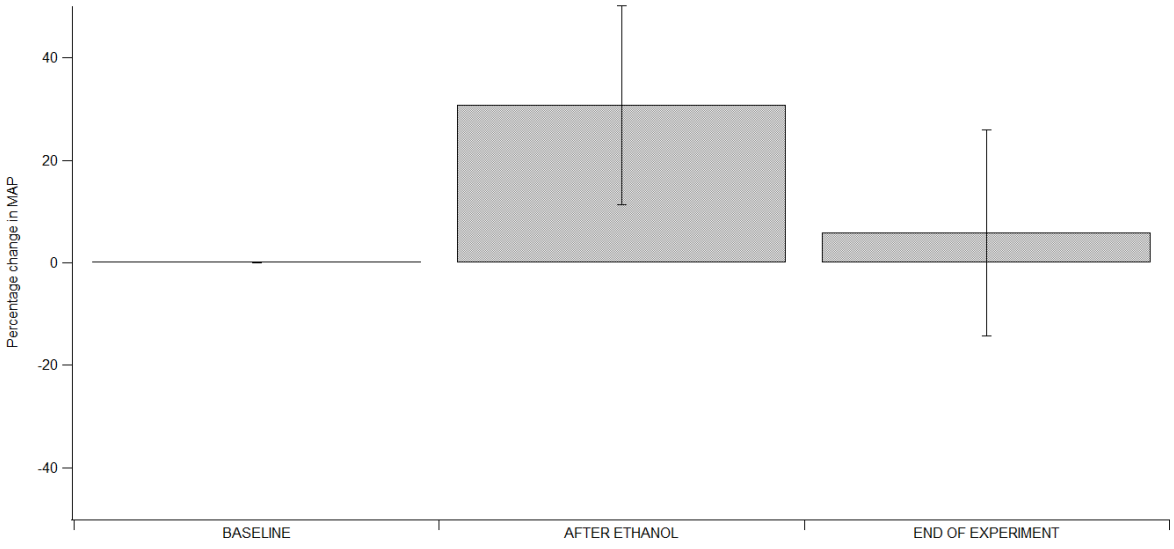


Fig.6: The control group showed an increase in MAP by 30.7percent (p=0.043)* after the administration of Ethanol from the baseline. At the end of 8 hours an increase of 5.8 (p=0.416) percent from the baseline was observed in the control group.

Percentage change in Mean Arterial Pressure (MAP):

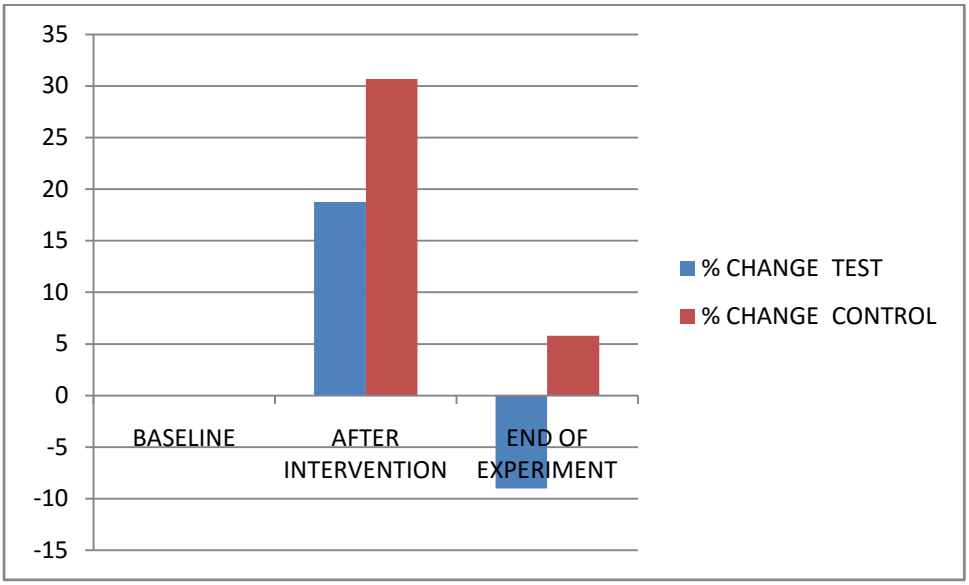


Fig.7: Shows the comparison between the percentage change in mean arterial pressures between test and control group. There was no significant difference between the test and the control groups either after intervention (p=0.564) or at the end of the experiment (p=0.564). (Mann Whitney U)

Change in heart rate:

The following graphs show the effect of intraperitoneal injection of Cleistanthin C on Heart Rate (HR) (Fig.8) as compared to that in control animals (Fig.9)

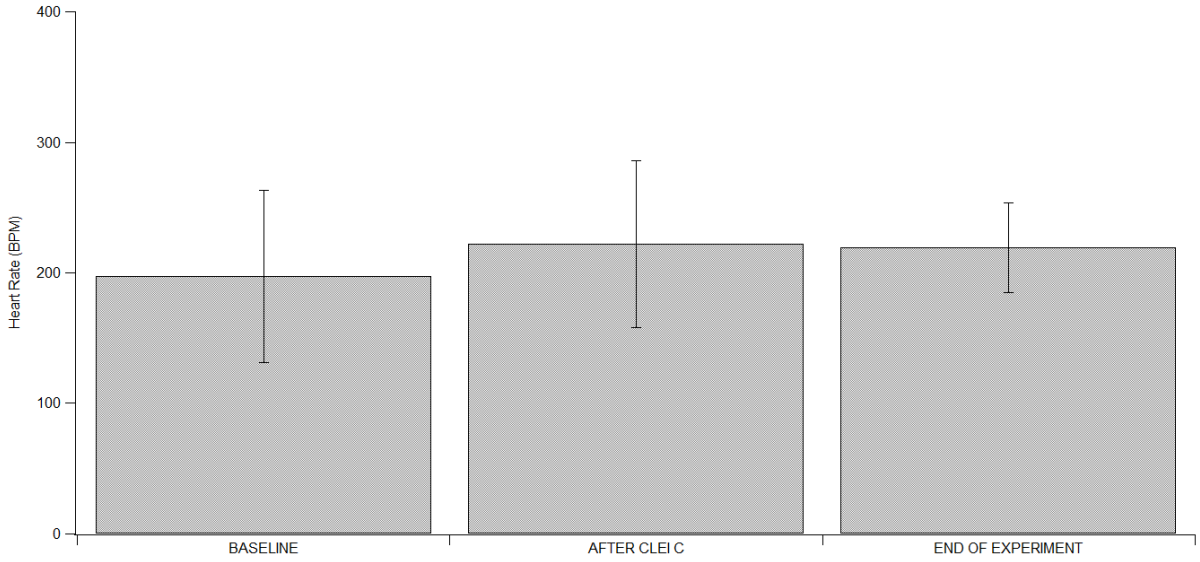


Fig.8: Effect of Intraperitoneal injection of Cleistanthin C on heart rate of rats. HR increased from 197±65.9 BPM to 222±64 BPM (p=0.068) before dropping to 219±34.6 BPM(p=0.5) at the end of 8 hours.

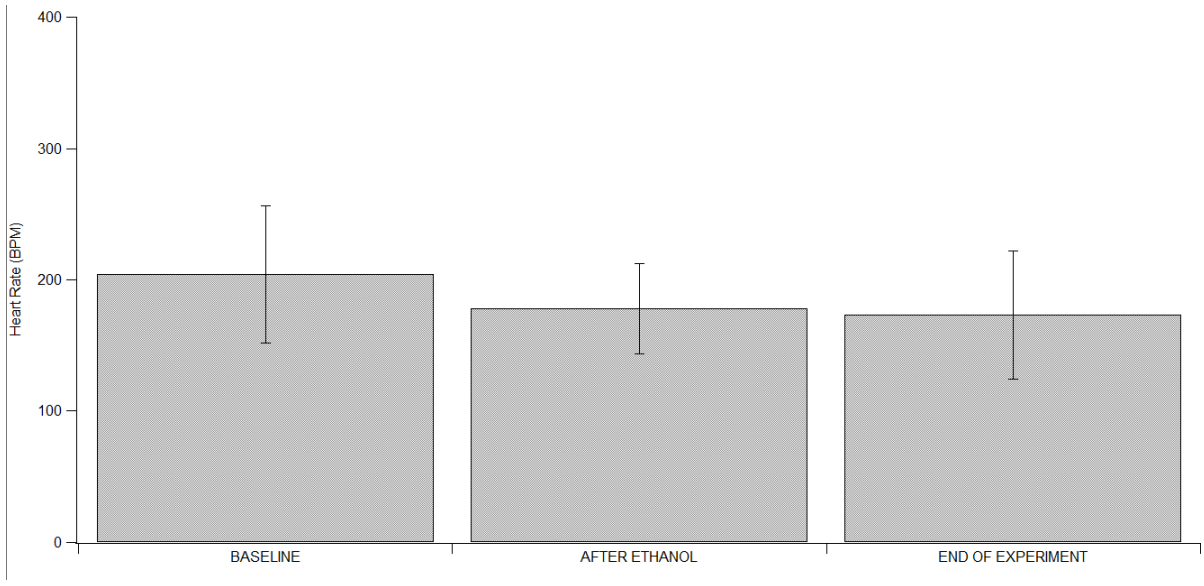


Fig.9: Effect of Intraperitoneal injection of 30% on heart rate of rats. HR decreased from 203±52.2 BPM to 177.8±34.5 BPM (p=0.5) before dropping further to 174.4±48.7 BPM (p=0.08) at the end of 8 hours.

Change in Systolic Blood Pressure (SBP):

The following graphs show the effect of intraperitoneal injection of Cleistanthin C on Systolic Blood Pressure (SBP) (Fig.10) as compared to that in control animals (Fig.11)

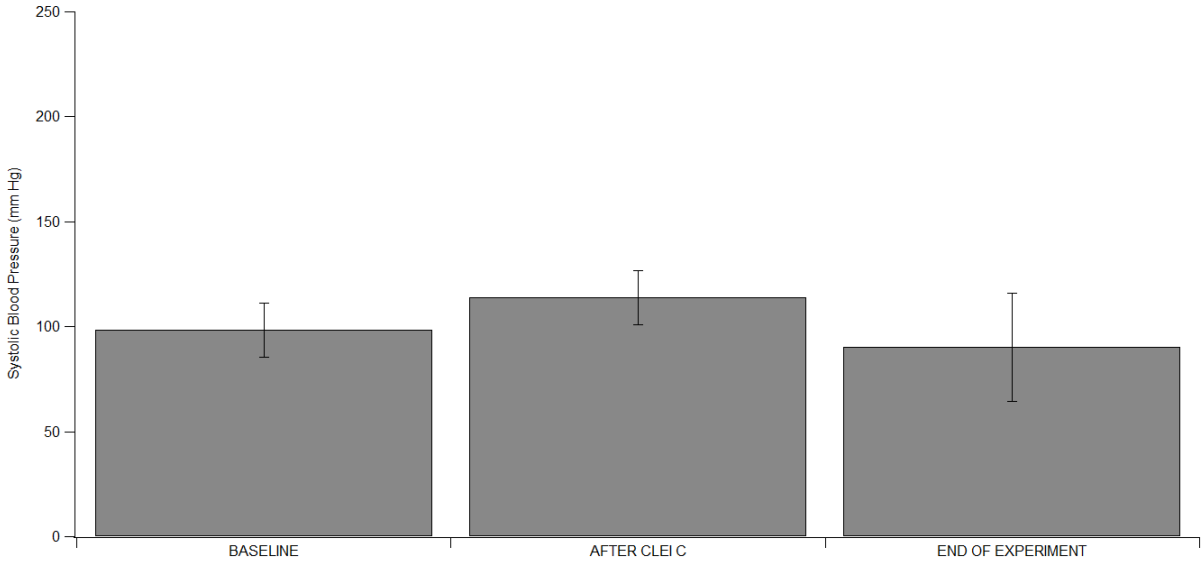


Fig. 10: Effect of Intraperitoneal injection of Cleistanthin C on systolic blood pressure of rats. SBP increased from 98.4±12.7 mm Hg to 114± 13.1 mmHg (p=0.043)* before dropping to 90.4±25.9 mmHg (p=0.345) at the end of 8 hours.

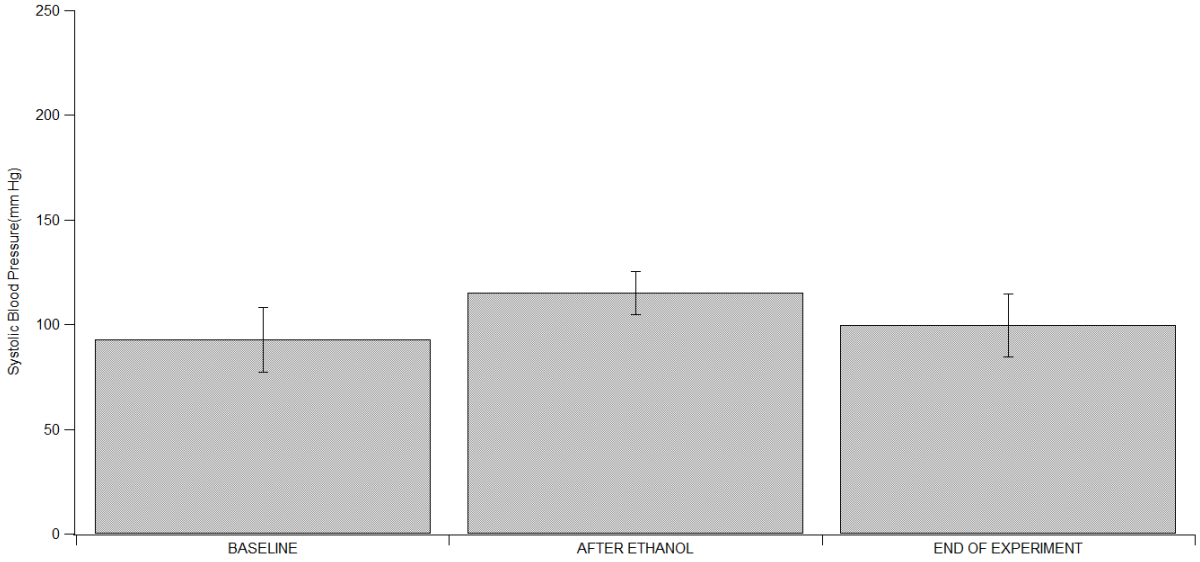


Fig. 11: Effect of Intraperitoneal injection of 30% ethanol on systolic blood pressure of rats. SBP increased from 92.6±15.3.6mm Hg to 115± 10.5 mmHg (p=0.043)* before dropping to 99.8±15.2 mmHg (p=0.224) at the end of 8 hours.

Changes in Diastolic Blood pressure (DBP):

The following graphs show the effect of intraperitoneal injection of Cleistanthin C on Diastolic Blood Pressure (DBP) (Fig.12) as compared to that in control animals (Fig.13):

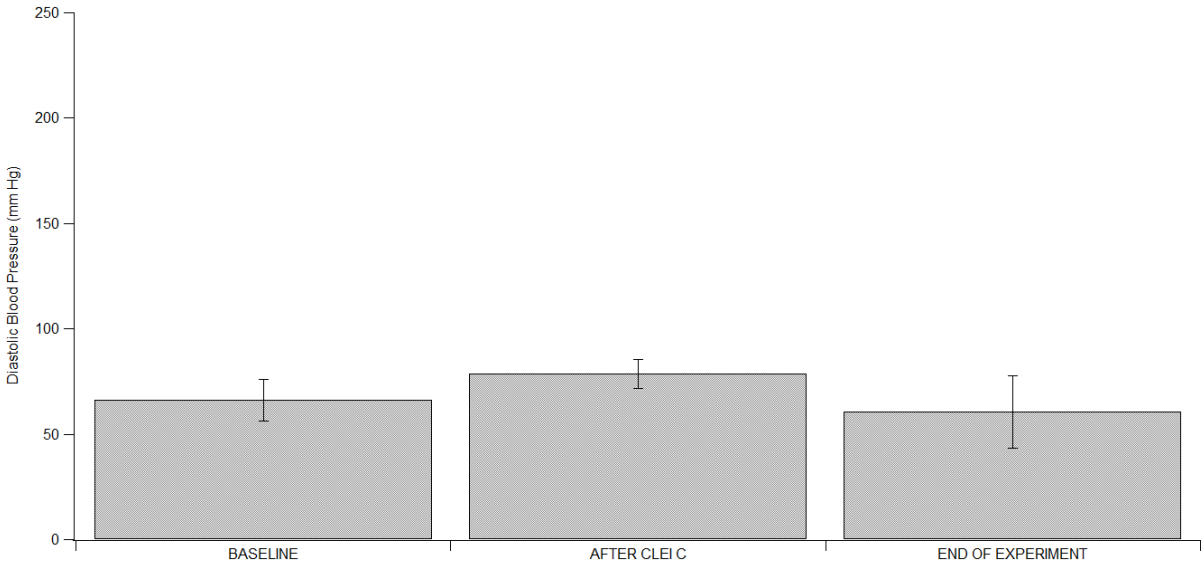


Fig. 12: Effect of Intraperitoneal injection of Cleistanthin C on diastolic blood pressure of rats. DBP increased from 66.2±9.7 mm Hg to 78.6± 6.7 mmHg (p=0.043)* before dropping to 60.4±17.2 mmHg (p=0.273) at the end of 8 hours.

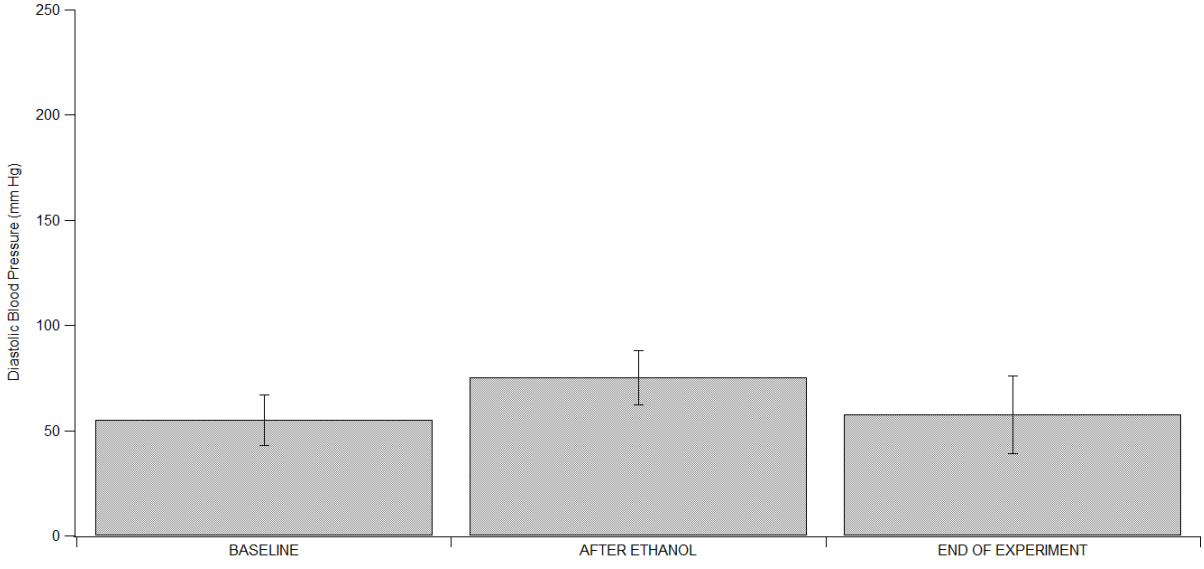


Fig. 13: Effect of Intraperitoneal injection of 30% ethanol on diastolic blood pressure of rats. DBP increased from 55±12.2mm Hg to 75.2±13.1 mmHg (p=0.043)* before dropping to 57.4±18.3 mmHg (p=0.893) at the end of 8 hours.

Changes in Pulse Pressure (PP):

The following graphs show the effect of intraperitoneal injection of Cleistanthin C on Pulse Pressure (PP) (Fig.14) as compared to that in control animals (Fig.15):

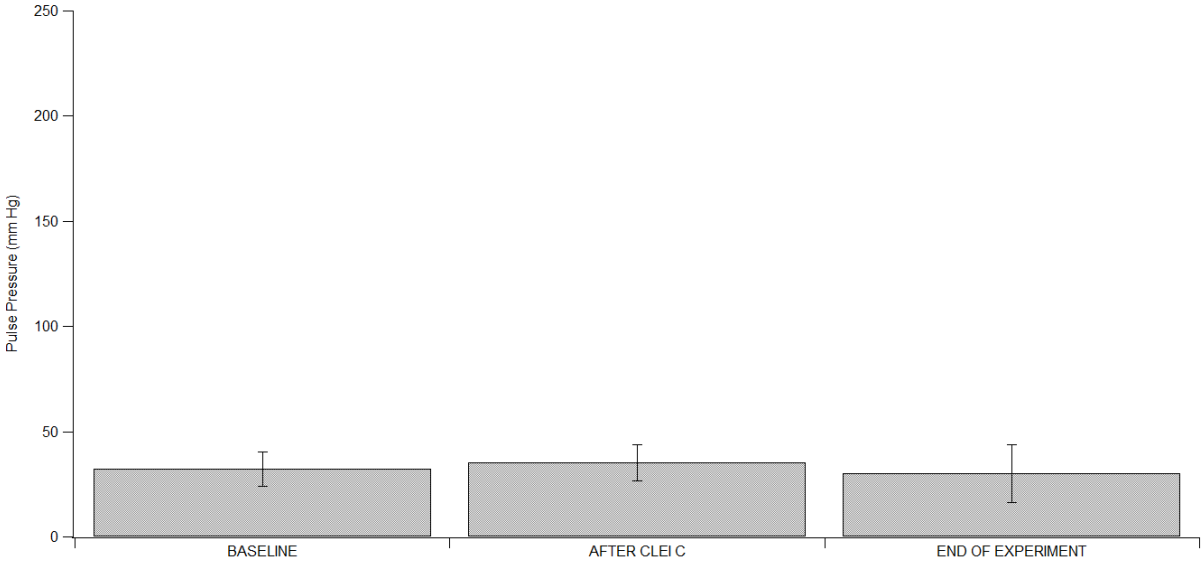


Fig. 14: Effect of Intraperitoneal injection of Cleistanthin C on pulse pressure of rats. PP increased from 32.2±8 mm Hg to 35.2±8.4 mmHg (p=0.068) before dropping to 30±13.9 mmHg (p=0.686) at the end of 8 hours.

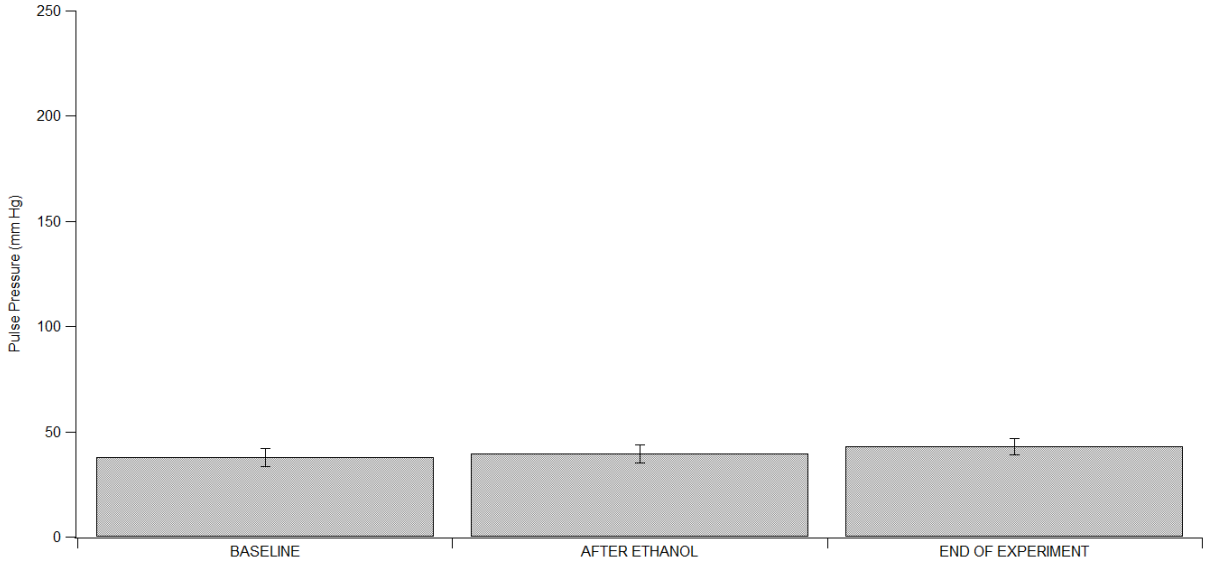


Fig. 15: Effect of Intraperitoneal injection of 30% ethanol on pulse pressure of rats. PP increased from 37.6±4.4mm Hg to 39.6± 4.2 mmHg (p=0.684) before increasing further to 43±3.8 mmHg (p=0.223) at the end of 8 hours.

INTRAVENOUS INTERVENTION GROUP:

The figures below show the representative pressure traces of test (Fig.16) and control (Fig.17) groups.

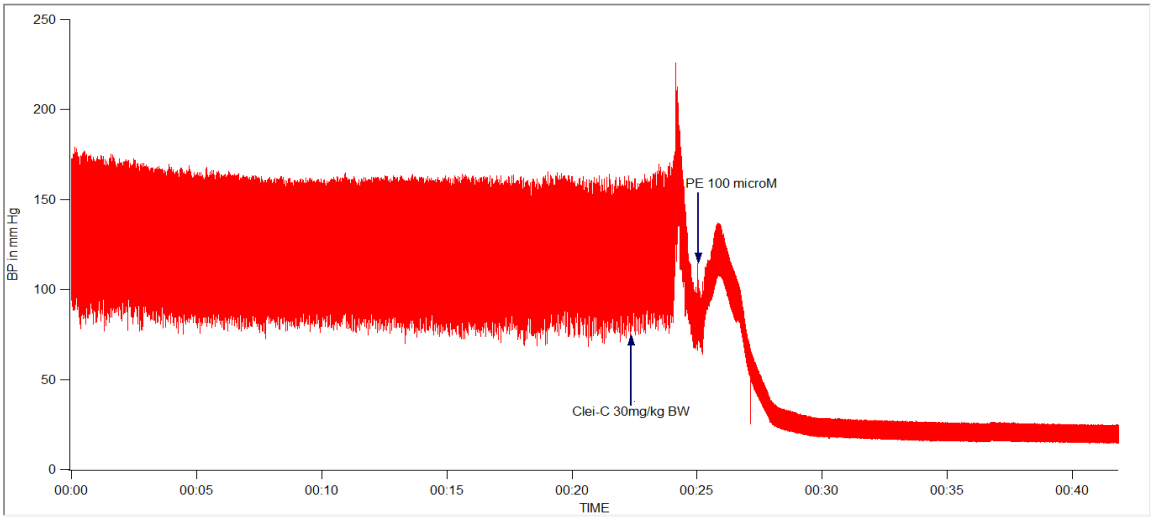


Fig.16: Effect of intravenous administration of Cleistanthin C followed by Phenylephrine on blood pressure of rats.

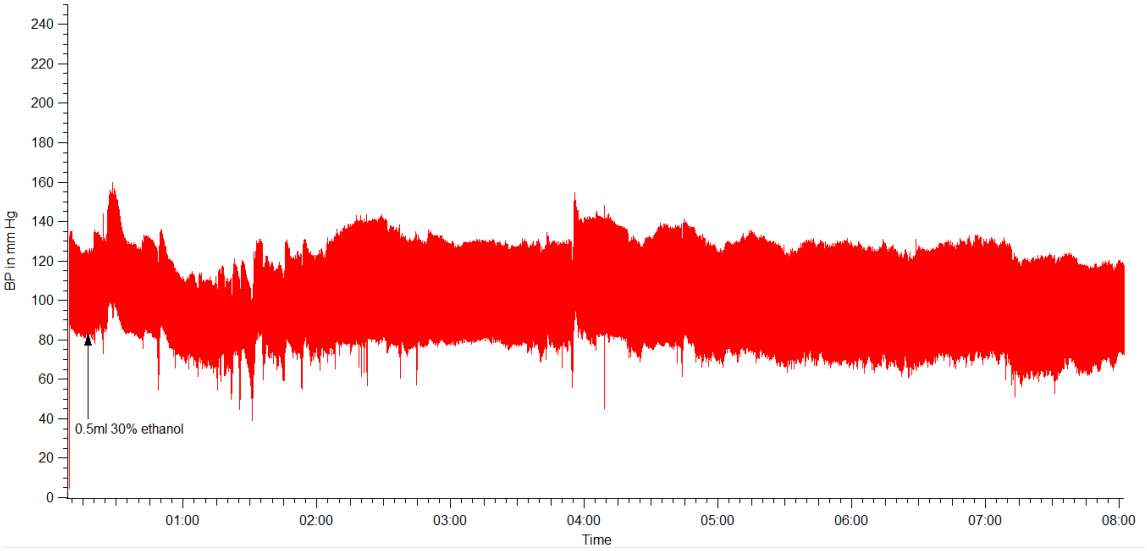


Fig.17: Effect of intravenous administration of 30%ethanol on blood pressure of rats.

Effect of intravenous administration of Cleistanthin C on various hemodynamic parameters:

	INTRAVENOUS INTERVENTION					
PARAMETERS		TEST			CONTROL	
	BASELINE	POST CLEISTANTHIN C	AFTER PE	BASELINE	POST ETHANOL	END OF EXPERIMENT
HEART RATE(BPM)	224±66.9	175.4±76.1	75.4±22.2	181.75±31	205±52.6	199.75±47
SYSTOLIC BP(mm Hg)	118±28.1	100.6±14.6	33±7.9	115±40.8	118±33.9	127.75±20.3
DIASTOLIC BP(mm Hg)	74±18.7	52.8±14.9	13.2±9	74±28.1	74±27	77.25±14.8
PULSE PRESSURE (mm Hg)	46.2±13.4	47.6±16.1	20.2±6.8	41±15.2	44.5±13.7	50.5±9.8
MEAN ARTERIAL PRESSURE(mm Hg)	87.8±19.7	63.8±12.9	19.6±7.8	87.75±32.1	88.75±28.9	94±15.8
% CHANGE IN MAP	0±0	-20.1±13.8	-76.4±13	0±0	3.78±26.8	17.68±47.5

Table 2: The above table shows the effect of intravenous injection of Cleistanthin C on various hemodynamic parameters. These include: 1.Heart Rate, 2. Systolic blood pressure, 3. Diastolic blood pressure, 4. Pulse pressure, 5. Mean arterial pressure and 6. Percentage change in mean arterial pressure.

EFFECT ON MEAN ARTERIAL PRESSURE:

The following graphs show the effect of intravenous injection of Cleistanthin C on Mean Arterial Pressure (MAP) (Fig.18) as compared to that in control animals (Fig.19)

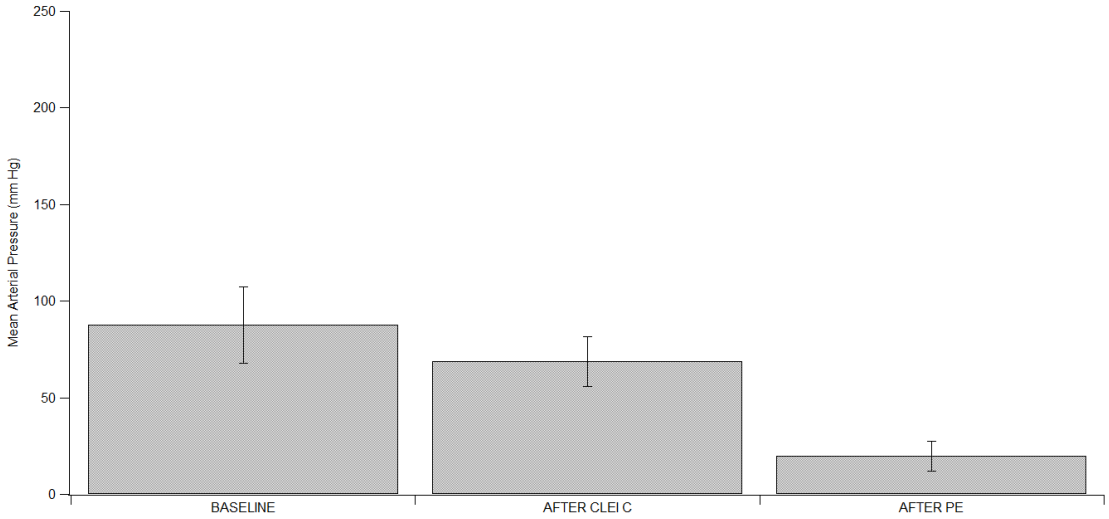


Fig.18: Shows the effect of intravenous injection of Cleistanthin C followed by Phenylephrine on Mean Arterial Pressure of rats. The MAP decreased from 87.8±19.7 mm Hg to 68.8±12.9 mm Hg (p=0.080) after Cleistanthin C administration which dropped further to 19.6±7.8 mm Hg (p=0.043)*.

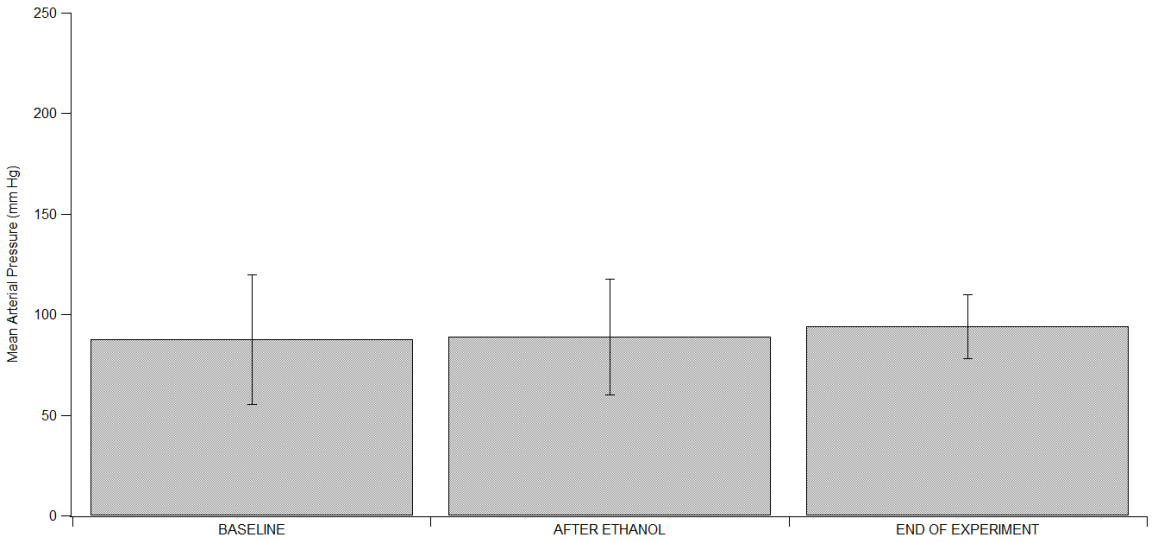


Fig.19: Shows the effect of intravenous administration of 30% ethanol on Mean Arterial Pressure of rats. The MAP changed from 87.75±32.1 mmHg to 88.75±28.9 mm Hg (p=1.00) finally reaching 94±15.8mm Hg (p=1.00) at the end of 8 hours.

PERCENTAGE CHANGE IN MEAN ARTERIAL PRESSURE:

The following graphs show percentage change in on Mean Arterial Pressure (MAP) after intraperitoneal injection of Cleistanthin C (Fig.20) as compared to that in control animals (Fig.21)

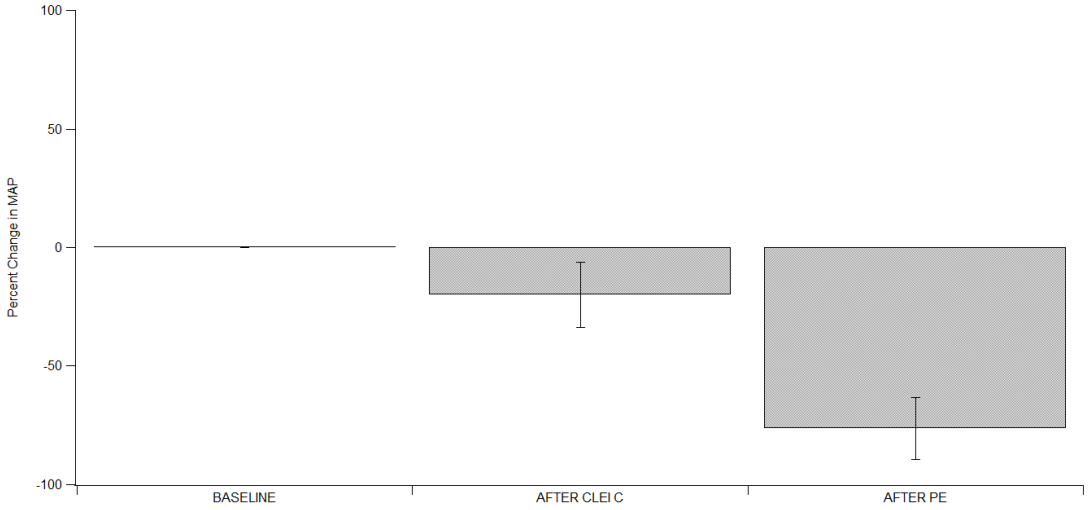


Fig.20: Shows the percentage change in mean arterial pressure test group. There was a percentage drop in blood pressure of 20.1±13.8 (p=0.080) in the test group after administration of Cleistanthin C which dropped further by 76.4±13percent (p=0.043)* after addition of Phenylephrine (PE) finally causing the death of the animal.

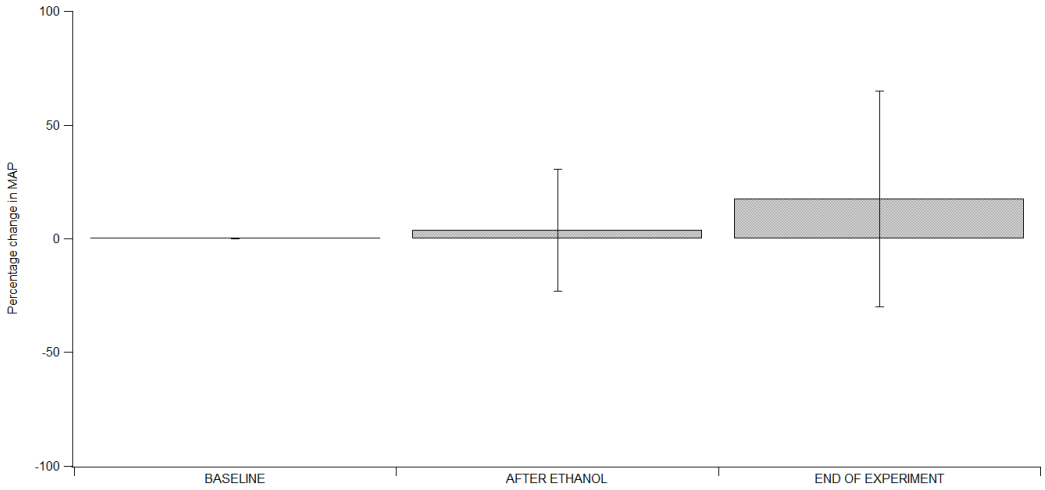


Fig.21: Shows the percentage change in mean arterial pressure control group. There was a percentage increase in blood pressure of 3.78±26.8 (p=0.715) in the test group after administration of Cleistanthin C which increased further by 17.68±47.5 per cent (p=0.465) from baseline at the end of 8 hours.

Percentage change in Mean Arterial Pressure (MAP):

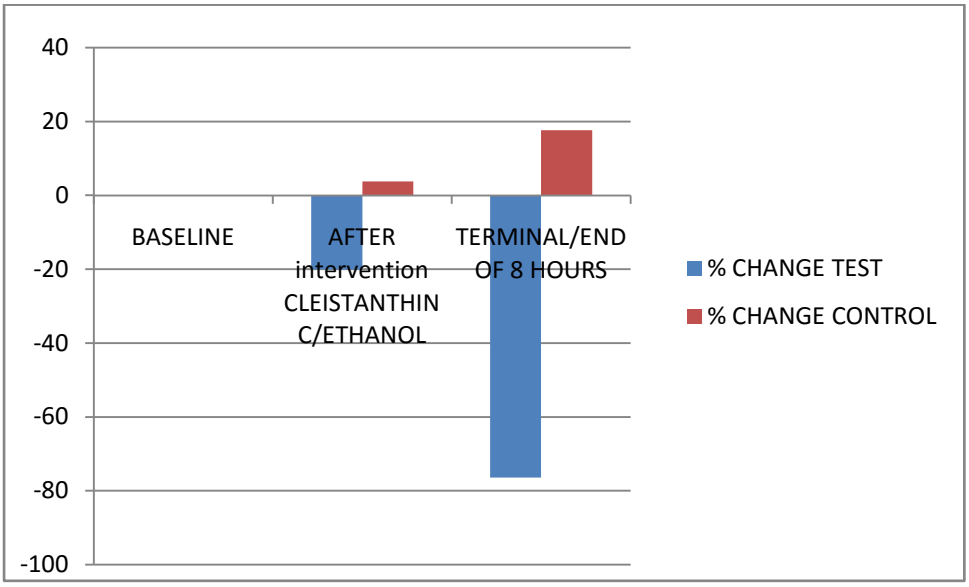


Fig.22: Shows the comparison between the percentage change in mean arterial pressures between test and control group. There was a significant difference in change in Mean Arterial Pressure between the test and the control groups after intervention (Cleistanthin C/ Ethanol) (p=0.043)* and even more at the end of the experiment (p=0.021)*. (Mann Whitney U)

Change in heart rate:

The following graphs show the effect of intravenous injection of Cleistanthin C on Heart Rate (HR) (Fig.23) as compared to that in control animals (Fig.24).

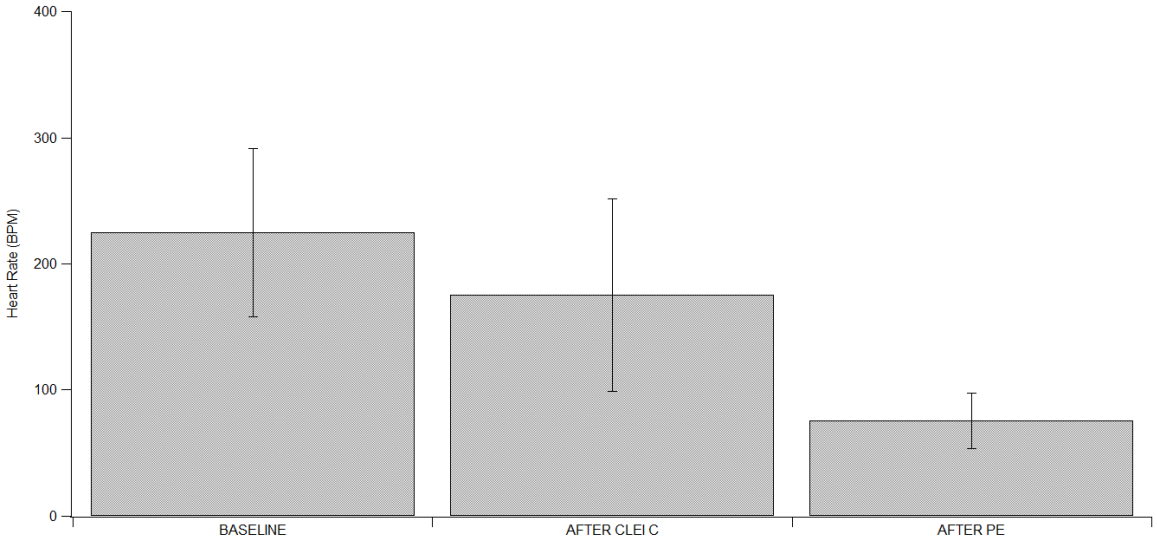


Fig.23: Effect of Intravenous injection of Cleistanthin C followed by Phenylephrine on heart rate of rats. HR decreased from 224.6±66.9 BPM to 175.4±76.1 BPM (p=0.345) after Cleistanthin C administration before dropping further to 75.4±22.2 BPM (p=0.043)* just before the death of the animal.

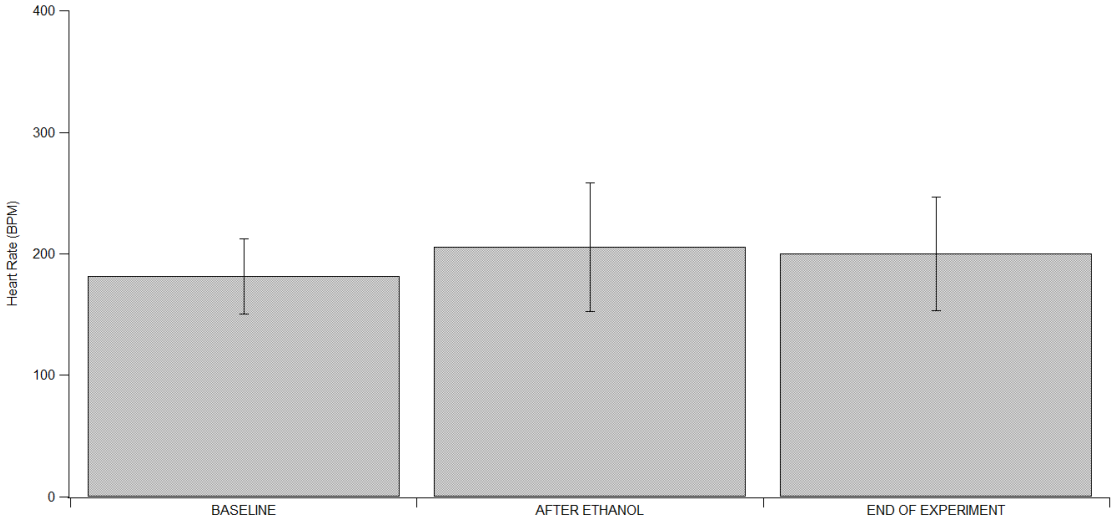


Fig.24: Shows the effect of intravenous administration of 30% ethanol on Heart Rate of rats. The HR changed from 181.75±31BPM to 205.5±52.6 BPM (p=1.00) finally reaching 199.75±47 BPM (p=0.715) at the end of 8 hours.

Change in Systolic Blood Pressure (SBP):

The following graphs show the effect of intravenous injection of Cleistanthin C on Systolic Blood Pressure (SBP) (Fig.25) as compared to that in control animals (Fig.26).

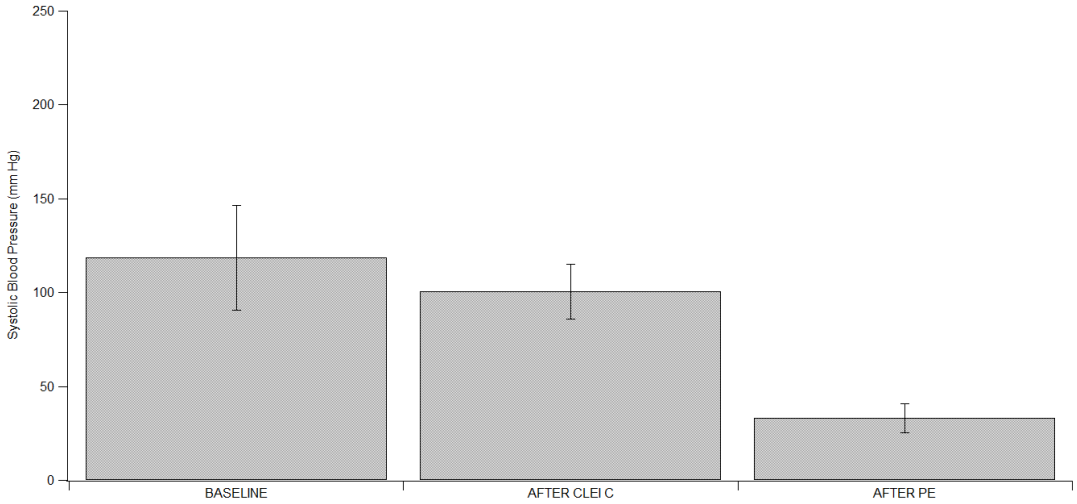


Fig. 25: Effect of Intravenous injection of Cleistanthin C followed by Phenylephrine on Systolic Blood Pressure of rats. SBP decreased from 118.4±28.1 mm Hg to 100.6±14.6 mmHg (p=0.080) after Cleistanthin C administration before dropping to 33±7.9 mmHg (p=0.043)* just before the death of the animal.

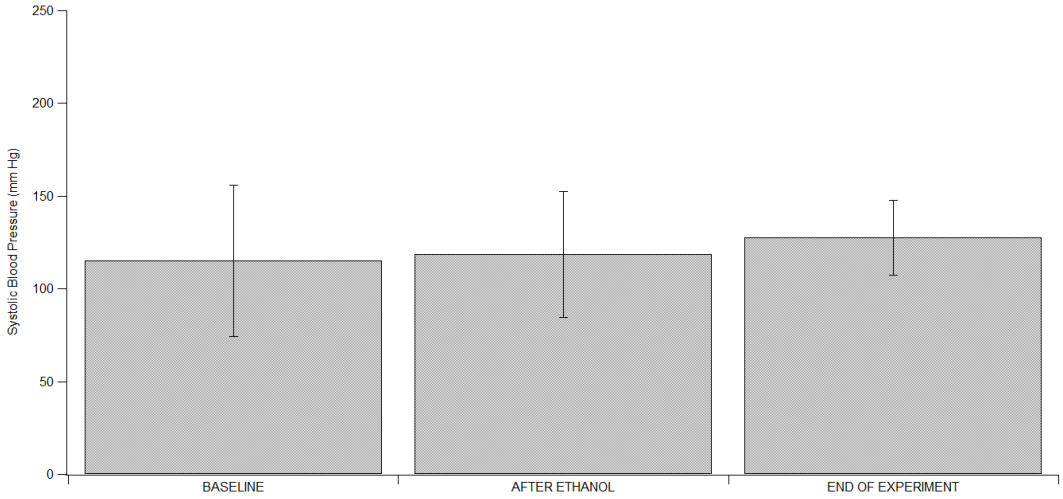


Fig.26: Shows the effect of intravenous administration of 30% ethanol on Systolic Blood Pressure of rats. The SBP changed from 115±40.8mm Hg to 118.5±33.9 mm Hg (p=1.00) finally reaching 127.75±20.3 mm Hg (p=0.715) at the end of 8 hours.

Change in Diastolic Blood Pressure (DBP):

The following graphs show the effect of intravenous injection of Cleistanthin C on Diastolic Blood Pressure (DBP) (Fig.27) as compared to that in control animals (Fig.28).

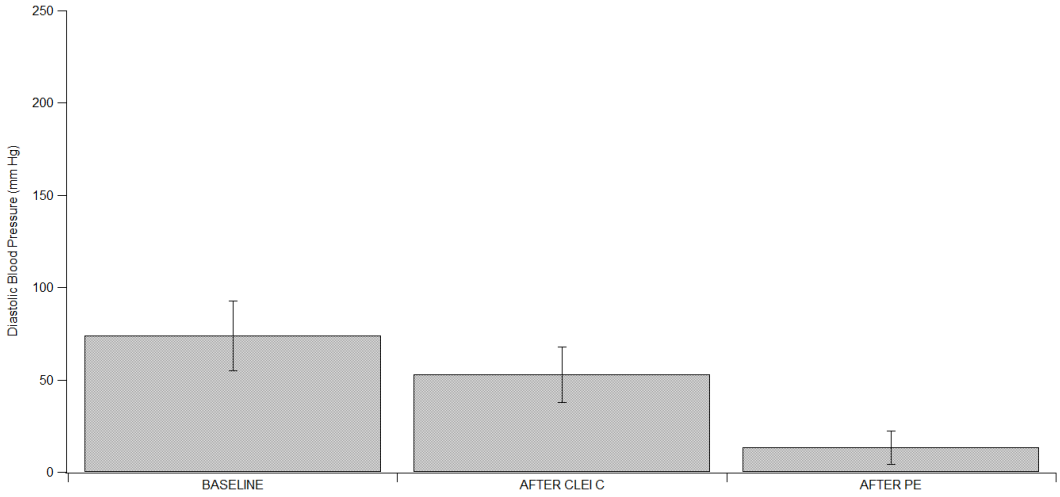


Fig. 27: Effect of Intravenous injection of Cleistanthin C followed by Phenylephrine on Diastolic Blood Pressure of rats. DBP decreased from 74±18.7 mm Hg to 52.8±14.9 mmHg (p=0.043)* after Cleistanthin C administration before dropping to 13.2±9 mmHg (p=0.042)* just before the death of the animal.

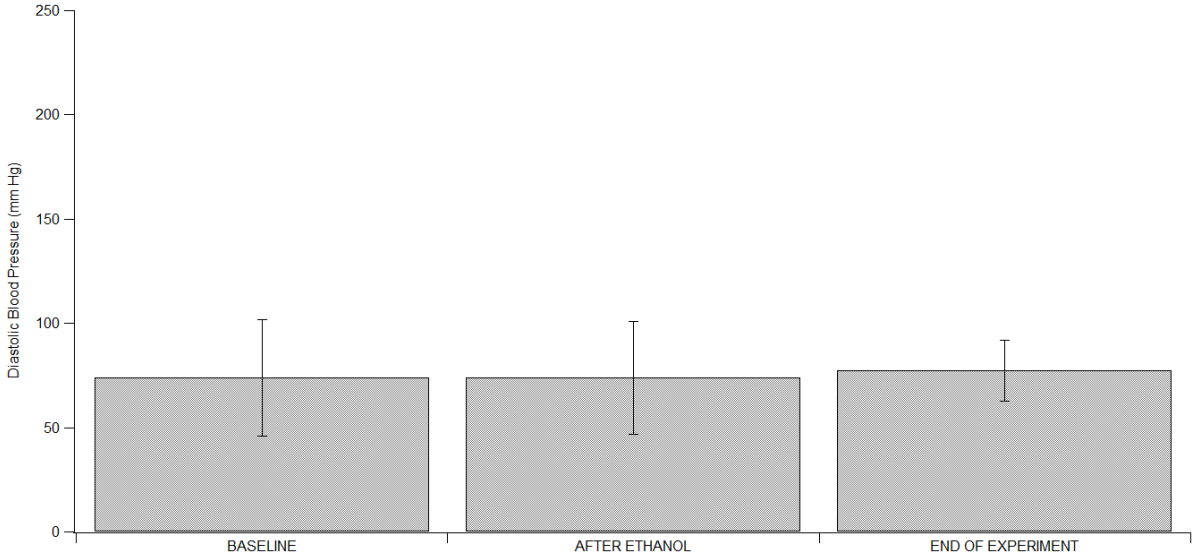


Fig.28: Shows the effect of intravenous administration of 30% ethanol on Diastolic Blood Pressure of rats. The DBP changed from 74±28.1mm Hg to 74±27 mm Hg (p=1.00) finally reaching 77.25±14.8 mm Hg (p=1.00) at the end of 8 hours.

Change in Pulse Pressure (PP):

The following graphs show the effect of intravenous injection of Cleistanthin C on Pulse Pressure (SBP) (Fig.29) as compared to that in control animals (Fig.30).

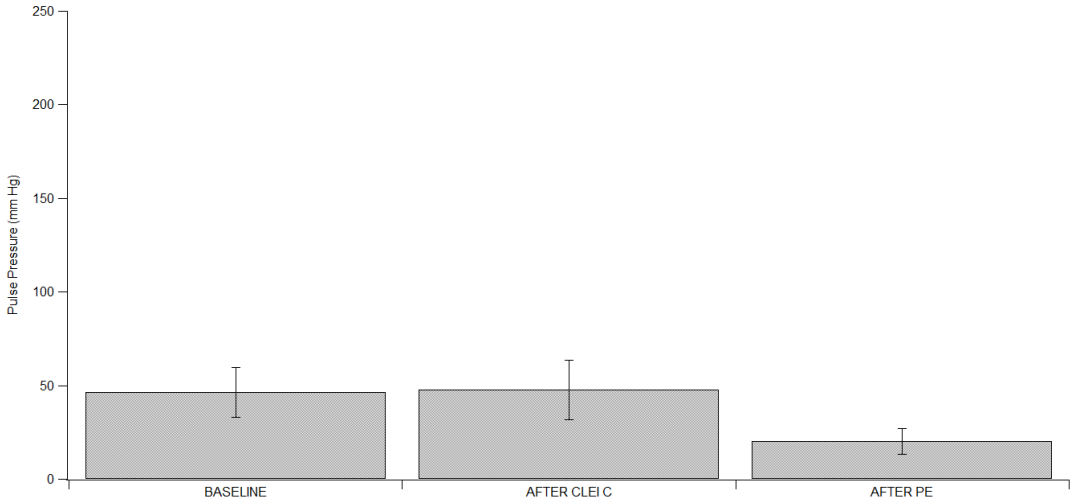


Fig. 29: Effect of Intravenous injection of Cleistanthin C followed by Phenylephrine on Pulse Pressure of rats. PP decreased from 46.2±13.4 mm Hg to 47.6±16.1 mmHg (p=0.713) after Cleistanthin C administration before dropping to 20.2±6.8 mmHg (p=0.043)* just before the death of the animal.

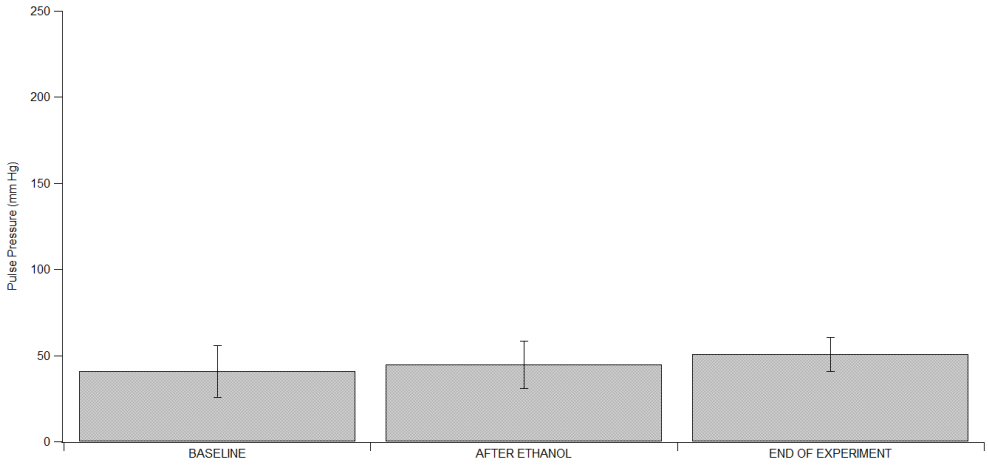


Fig.30: Shows the effect of intravenous administration of 30% ethanol on Pulse Pressure of rats. The PP increased from 41±15.2 mm Hg to 44.5±13.7 mm Hg (p=0.465) finally reaching 50.5±9.8 mm Hg (p=0.141) at the end of 8 hours.

Discussion

As we can see from the results, the changes in Blood pressure seen in the **intravenous** intervention group are much **quicker and dramatic** as compared to the group which received intraperitoneal injection.

Even though there was a drop in mean arterial pressure in both the intervention groups, the drop in the mean arterial pressure in **the intraperitoneal** intervention group was **not enough to cause death** in the animals even after 8 hours.

We shall discuss each intervention groups separately.

Intraperitoneal intervention group:

The group which received intraperitoneal injection of Cleistanthin C **did not show mortality** even at a dose 5 times the lethal dose (LD_{100}) determined previously in our laboratory.

Even after 8 hours the mean arterial pressure held at values which **neither caused mortality, nor required any intervention** with vasopressor drugs. The rats were finally sacrificed at the end of 8 hours.

In our pilot study we had noticed a slow and steady decrease in blood pressure over time. That decrease was probably due to dehydration due to constant loss of fluid through the open surgical wound at the cannulation site in the neck of the rats over the long duration of the experiment. However, once we started administration of adequate maintenance fluid, the animals held their blood pressure till the end of the experiment.

The results above show, an **increase in mean arterial pressure** on administration of **Cleistanthin C as well as in the control group**. This increase in the mean arterial pressure may be attributed to a transient increase in the volume of fluid administered.

By the **end of 8 hours**, the mean arterial pressure reaches a **level below the baseline** however, this drop is **not significant** ($p=0.225$). The mean arterial pressure in the control group although decreased, remained significantly high ($p=0.043$) at the end of 8 hours.

The **heart rate did not show any significant change** in both groups either post intervention or at the end of the experiment. This is not surprising as the changes in the mean arterial pressure were not enough to activate any reflexes.

The **systolic blood pressure**, showed a **significant increase on administration of Cleistanthin C** which dropped slightly below the baseline at the **end of 8 hours**, the change being statistically **non significant**. The control group showed similar changes in the systolic blood pressure.

The changes in the **diastolic blood pressure** were similar to those seen in the systolic blood pressure with a **significant increase post intervention** in both test as well as control groups. At the end of 8 hours the diastolic blood pressure dropped to levels not significantly different from the baseline in both groups.

The pulse pressure remained fairly constant and did not show any significant change either in test or the control group.

A probable reason for the slow changes in the intraperitoneal intervention group may be slow absorption of the drug by this route as compared to the intravenous route.

Another reason for this kind of changes may be, incomplete absorption of the compound with most of the compound precipitating in the peritoneal cavity itself.

It is because of these reasons we decided to administer the drugs intravenously.

Intravenous intervention group:

The **mortality in the intravenous group was 100%** in the test group as compared to 0% in the control group with the minimal lethal dose (LD_{100}).

The rats in the test group died between 20 minutes to 2 hours of administration of the minimal lethal dose intravenously. In the control group all the rats survived up to 8 hours after which they had to be sacrificed.

The rats in the control group managed to maintain stable hemodynamic parameters throughout the course of the experiment.

On administration of **Cleistanthin C**, there was a **decrease in the mean arterial pressure**. On administration of **Phenylephrine**, the mean arterial pressure **dropped precipitously** after a transient increase to levels low enough to cause the death of the animal. The **control animals**, who received only 30% Ethanol (vehicle), the mean arterial pressure, remained virtually **unchanged**.

When we look at the percentage change in mean arterial pressure, while the test rats showed a significant drop of 76% at the end of the experiment, the control group showed an insignificant increase if around 18% at the end of experiment.

When we compare the change in the mean arterial pressures between test and control animals, there is significant difference between the changes in test and control groups both immediately after the intervention **(p=0.043)** as well as at the end of the experiment **(p=0.021)**.

There was a **moderate decrease in heart rate** on administration of Cleistanthin C which **decreased further to significantly low levels** on administration of Phenylephrine. The control animals however did not show any significant change heart rate.

The **systolic blood pressure** too, showed a **moderate decrease on administration of Cleistanthin C** but there was a **significant drop on administration of Phenylephrine**. The control animals did not show any significant change in systolic blood pressure at any point during the experiment.

The **diastolic blood pressure** showed a slightly different profile. On **Cleistanthin C administration, there was a significant decrease** in diastolic blood pressure. This dropped further on Phenylephrine administration. In the **control group** the diastolic blood pressure remained **very stable** both after administration of ethanol **(p=1.00)** as well as at the end of the experiment **(p=1.00)**.

The **pulse pressure** did **not change on administration of Cleistanthin C**. However, there was a **significant decrease** in the pulse pressure on administration of **Phenylephrine.** The control group showed no significant change in the pulse pressure.

As we can see, intravenous intervention gives us a more obvious picture as compared to intraperitoneal method.

We can see from the above results, that while the administration of **Cleistanthin C alone** caused only a **moderate decrease in the mean arterial pressure, systolic**

blood pressure and heart rate, it caused a **significant decrease in the diastolic blood pressure** but practically **no change in the pulse pressure**.

We know that the total peripheral resistance is a stronger determinant of diastolic blood pressure than of systolic blood pressure or pulse pressure, it is reasonable to conclude that **Cleistanthin C works primarily at the levels of arterioles**.

On administration of **Phenylephrine** to the above rats, we see **significant decrease in all parameters** namely mean arterial pressure, systolic blood pressure, diastolic blood pressure, pulse pressure as well as heart rate till it led to death of the animal.

This result indicates to an additional distention at the level of large vessel. The **addition of Phenylephrine** in the presence of Cleistanthin C probably causes an **increase in compliance at the level of the large arteries** along with the previously existing decreased peripheral resistance. This increase in compliance explains the decrease in the systolic blood pressure, diastolic blood pressure and pulse pressure(28).

The decreased heart rate could be a direct effect of Cleistanthin C on the heart or could be secondary to a respiratory failure as reported in earlier studies(29). It may also be a primary bradycardia . It must be noted that in our experiments the ECG did not show any rhythm abnormalities other than bradycardia. There was a decrease on respiratory rate. However the decrease on the heart rate and the decrease in respiratory rate start almost at the same time, hence it is difficult to comment if the decreased heart rate is secondary to decrease in the respiratory rate.

Now the question arises, how can a vasoconstrictor drug like Phenylephrine cause vasodilatation?

In another study in our department, we have seen that alpha agonists, under specific conditions like high nitric oxide environment can actually produce vasodilatation. (27)

This may explain why the shock developed by *Cleistanthus collinus* poisoning is refractory to treatment. Since vasopressors form the main line of management for shock, it is possible that by administering them to victims of *Cleistanthus collinus* poisoning we may actually worsen the shock. Management of shock with other modalities such as fluid management may probably improve the prognosis.

Another area of exploration would the role of alpha adrenergic blockers in this kind of situation.

Conclusion

In conclusion, this study shows that Cleistanthin C causes drop in blood pressure of rats.

This dissertation also shows that Cleistanthin C alone causes a decrease in peripheral resistance by acting at the level of arterioles.

Administration of Phenylephrine to rats pre administered with Cleistanthin C probably causes dilatation of capacitance vessels thereby worsening the shock and may not be the ideal modality of management.

Absorption Cleistanthin C through intraperitoneal route is slow and incomplete hence it is not a good route of administration.

Limitations

Intraperitoneal route of administration is not reliable

A larger sample size would have yielded more accurate results.

Effect of Cleistanthin C alone (without vasopressor drug) needs to be explored.

The conclusions above could be proved better if changes in resistance and capacitance could be directly measured.

Role of alpha blockers needs to be further explored.

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OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA.

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDIN)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

March 19, 2015

Dr. Sajal Clarence Singh
PG Demonstrator
Department of Physiology
Christian Medical College, Vellore 632 002

Sub: **Fluid Research Grant Project:**
To observe the effect of Cleistanthin C on blood pressure of rats and the effect of various vaso pressors agents if hypotension occurs.
Dr. Sajal Clarence Singh, Physiology, Dr. Sathya Subramani, Physiology, CMC, Vellore.

Ref: IRB Min No. 9337 [OTHER] dated 03.03.2015

Dear Dr. Sajal Clarence Singh,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "To observe the effect of Cleistanthin C on blood pressure of rats and the effect of various vaso pressors agents if hypotension occurs." on March 3rd 2015.

CHRISTIAN MEDICAL COLLEGE VELLORE INDIA

The Committees reviewed the following documents:

1. IRB Application format
2. Curriculum Vitae' of Drs. Sajal Clarence Singh, Sathya Subramani
3. No of documents 1 – 2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on March 3rd 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS M Ch	Professor, Neurological Sciences, CMC, Vellore.	Internal, Clinician

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002.
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Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho, MS Ortho, DNB Ortho
Chairperson, Research Committee & Principal

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MD., MNAMS., DNB (Endo), FRACP (Endo), FRCP (Glas) (EDIN)
Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Rajesh Kannangai	MD, Ph D.	Professor & In-charge Retrovirus Laboratory (NRL under NACO), Department of Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Dr. Vivek Mathew	MD (Gen. Med.) D.M (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Dept. of Biostatistics, CMC, Vellore	Internal, Statistician
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB, Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mrs. Pattabiraman	B. Sc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Denise H. Fleming	B. Sc (Hons), PhD	Honorary Professor, Clinical Pharmacology, CMC, Vellore	Internal, Scientist & Pharmacologist
Dr. Anuradha Rose	MBBS, MD	Assistant Professor, Community Health	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Legal Expert, Vellore	External, Legal Expert
Rev. Joseph Devaraj	B. Sc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Jayaprakash Muliyil	BSC, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, Vellore	External, Scientist & Epidemiologist
Mrs. Sheela Durai	M Sc Nursing	Addl. Deputy Nursing Superintendent, Professor of Nursing in Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

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Deputy Chairperson
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson, IRB, Member Secretary (Ethics Committee), IRB	Internal, Clinician

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html

Fluid Grant Allocation:

A sum of 52,740/- INR (Rupees Fifty Two Thousand Seven Hundred and Forty only) will be granted for 2 years. The funds will be released only after the IAEC approval of the study.

Yours sincerely

Dr. Alfred Job Daniel
Chairperson, Research Committee & Principal
Institutional Review Board
Christian Medical College, Vellore

Cc: Dr. Sathya Subramani, Physiology, CMC, Vellore.

IRB Min No: 9337 [OTHER] dated 03.03.2015

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INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE

Dr. Alfred Job Daniel
Principal and Chairman
email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
email: vinayoommen@cmcvellore.ac.in

11th April 2015

To
Dr. Sajal Clarence Singh
PG Demonstrator
Physiology
CMC Vellore

Dear Dr. Sajal Clarence Singh

Your research proposal titled **"To observe the effect of Cleistanthin C on blood pressure of rats and the effect of various vaso pressors agents if hypotension occurs."** has been reviewed by the Institutional Animal Ethics Committee (IAEC) on 30th March 2015.

After discussion, **12 wistar rats for year I and 24 wistar rats for year II** have been approved for the study

Location of experiments : **College Animal House**

The IAEC approval number for the study is **7/2015**

You are required to maintain all records as per form D, ensure humane treatment of animals and submit a **final report** to the IAEC. If an extension or the use of new animals after the sanctioned period is required, a **progress report** must be submitted to the IAEC with a request for new animals.

With best wishes,
Yours sincerely,

Dr. Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc:
Dr. Vinay Timothy Oommen
Secretary, IAEC